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IDENTIFICATION OF GENES DIFFERENTIALLY EXPRESSED IN *P. pinea* AND *P. pinaster* AFTER INFECTION WITH THE PINE WOOD NEMATODE (PWD) USING THE SSH TECHNIQUE

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under the supervision of

Marta Wilton de Vasconcelos

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RESUMO

O objectivo deste estudo consistiu na comparação da resposta à infecção com o nemátode da madeira do pinheiro (NMP) *Bursaphelenchus xylophilus* entre *Pinus pinaster* e *Pinus pinea* no estádio inicial da doença, três horas após inoculação. O NMP é o agente causal da doença da madeira do pinheiro, tem em Portugal como hospedeiro principal o pinheiro bravo, *P. pinaster,* e tem como vector *Monochamus galloprovincialis*. Curiosamente, esta doença parece não afectar a espécie *P. pinea* e vários factores podem estar na origem desta diferença de susceptibilidade.

Nas primeiras horas após uma infecção por patogénios, a árvore desenvolve uma resposta hipersensitiva, que consiste na produção de proteínas de resistência, entre outros compostos cruciais de defesa. Actualmente, não há estudos que descrevam os efeitos da infecção do nemátode nas plantas a um estádio inicial da doença.

No que diz respeito aos efeitos fisiológicos e metabólicos nas plantas, verificou-se uma diminuição de 10,9% no teor de água em *P. pinaster* (por comparação com árvores inoculadas com água) e 6,7% em *P. pinea*; relativamente à concentração de clorofilas, uma diminuição para cerca de metade do valor controlo foi registado para ambas as espécies, mas mais pronunciadamente em *P. pinaster*. Um estudo exploratório com GC também foi efectuado, que demonstrou que os compostos voláteis produzidos permitem uma clara diferenciação entre espécies, mas não entre inoculação controlo *vs*. nemátode.

No que diz respeito aos efeitos da doença ao nível da transcrição de genes, utilizou-se a técnica de SSH para identificar ESTs em *P. pinaster* e *P. pinea* inoculados com NMP. Os ESTs foram isolados, clonados, sequenciados e identificados usando BlastN e BlastX, e indicam claramente que no estádio inicial da doença existe a activação de uma resposta de defesa ao nível molecular relacionada principalmente com o stress oxidativo, produção de lenhina e de etileno e regulação pós-transcripcional dos ácidos nucleicos. Finalmente, 58% das sequências isoladas não estão ainda descritas, o que mostra a falta de informação genómica actual existente para o pinheiro.

ABSTRACT

The purpose of this study was to compare the response to infection with the pine wood nematode (PWN) *Bursaphelenchus xylophilus* between *Pinus pinaster* and *Pinus pinea* at an initial stage of the disease, three hours after inoculation. The PWN is the causal agent of pine wilt disease, in Portugal its main host is the maritime pine, *P. pinaster*, and its vector is *Monochamus galloprovincialis*. Interestingly, this disease does not seem to affect the species *P. pinea* and several factors could be behind this difference in susceptibility.

At the first hours following infection by pathogens, the tree develops a hypersensitive response, which includes the production of resistance proteins, among other compounds crucial to the defence mechanism. Currently there are no studies that describe the effects of nematode infection in plants at an early stage of the disease.

With regards to the physiological and metabolic indicators of the disease response in the plants, there was a decrease of 10.9% of water content in *P. pinaster* (compared with trees inoculated with water) and 6.7% in *P. pinea*; in the study of total chlorophyll concentration, a decrease to about half of the control value was recorded for both species, but more pronounced *in P. pinaster*. An exploratory study was also made with GC, which showed that the volatile compounds produced were distinctive between species, but not between control *vs*. nematode-inoculated plants.

With regards to the effects of the disease at a transcriptional level, the SSH technique was utilized to identify ESTs in *P. pinaster* and *P. pinea* when inoculated with NMP. ESTs were isolated, cloned, sequenced and identified using BlastN and BlastX, and clearly indicated that at an initial stage of the disease there is activation of a defence response at a molecular level, mainly related to oxidative stress, production of lignin and ethylene and post-transcriptional regulation of nucleic acids. 58% of the isolated sequences are not yet described, which shows the lack of genomic information currently available for pine.

ACKNOWLEDGEMENTS

I would like to share my deepest thanks to my supervisor, Drª Marta Vasconcelos, for accepting and welcoming me in her research group and for sharing with me her vast knowledge. Without her guidance, I wouldn't be able to finish this work.

To all Plantech group, I leave here my sincere appreciation, for all the support, knowledge and brainstorming that we've shared with each other.

I am thankful to my parents, Carlos and Otília, and to my sister, Cristina, for all your love and for helping and supporting me through my MSc.

Finally, I'd like to thank my boyfriend Vasco, for helping me to be resilient in my academic life, for never letting me quit and for being by my side always, no matter what.

CONTENTS

ABBREVIATIONS

* *B.*	*Bursaphelenchus*
* cDNA	Complementary deoxyribonucleic acid
* CIA	Chloroform isoamyl alcohol
* cm	Centimetres
* CTAB	Cetyl trimethylammonium bromide
* D	Subtracted Driver *P. pinea*
* Dai	Days after inoculation
* DNA	Deoxyribonucleic acid
* dNTP	Deoxyribonucleotide triphosphate
* ds	Double stranded
* PWD	Pine wilt disease
* PWN	Pine Wood Nematode
* EDTA	Ethylenediamine tetraacetic acid
* EF1α	Elongation Factor 1 alpha
* *E. coli*	*Escherichia coli*
* EST	Expression sequence tags
* g	Grams
* GC-MS	Gas Chromatography – Mass Spectrometry
* h	Hours
* Hai	Hours after inoculation
* HCl	Hydrochloric acid
* HR	Hypersensitive reaction
* HS-SPME	Headspace solid-phase microextraction
* kb	Kilobase
* LiCl	Lithium chloride
* *M.*	*Monochamus*
* M	Molar (10^6)
* mg	Milligrams
* mRNA	Messenger ribonucleic acid
* miRNA	Micro ribonucleic acid
* min	Minutes
* ml	Millilitres

* NaCl	Sodium chloride
* nm	Nanometres
* o.n.	Overnight
* *P.*	*Pinus*
* PAL	Phenylalanine ammonia lyase
* PCA	Principal component analysis
* PCR	Polymerase chain reaction
* PROLUNP	Programa Nacional de Luta Contra o Nemátode da Madeira do Pinheiro
* PVP	Polyvinylpyrrolidone
* *P. pinaster* + H2O	*P. pinaster* inoculated with water
* *P. pinaster* + HF	*P. pinaster* inoculated with B. xylophilus strain HF
* *P. pinea* + H2O	*P. pinea* inoculated with water
* *P. pinea* + HF	*P. pinea* inoculated with B. xylophilus strain HF
* QTL	Quantitative trait loci
* RNAi	Interference ribonucleic acid
* rpm	Revolution per minute
* RRM	RNA Recognition Motif
* SDE	Simultaneous distillation-extraction
* ss	Single stranded
* SSH	Suppression subtractive hybridization
* STE	Sodium-Tris-EDTA
* T	Subtracted Tester *P. pinaster*
* TE	Tris-EDTA
* TRX	Thioredoxin
* Ud	Unsubtracted Driver *P. pinea*
* Ut	Unsubtracted Tester *P. pinaster*
* XET	Xyloglucan endotransglycosylase
* X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside
* μ	Micro (10^{-6})

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Percentages of genes in each category are also presented. (a) These genes had no significant hits in BlastN and BlastX searches of NCBI. (b) These genes matched proteins annotated as 'unknown protein'.

* Figure 13 – Conserved domain information about the 15 genes annotated as "unknown protein" in NCBI databases.

* Figure 14 – Genus characterization of induced genes. (a) These genes had no significant hits in BlastN and BlastX searches of NCBI.

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1. INTRODUCTION

"Pine wilt disease results from a multitude of complicated, biological organisms, that include the pathogen, a host, an insect vector and climatic conditions." (Zhao *et al*., 2008). *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle, 1970 is a pinewood nematode (PWN) and is the causal agent of pine wilt disease (PWD) (Figure 1) (Nickle *et al*., 1981). The genus, *Bursaphelenchus* Fuchs 1937 includes almost 90 species and is a member of the Family Aphelenchoididae; nematodes belonging to this genus can be mycophagus, plant parasitic or both, and *B*. *xylophilus* falls in the later category (Zhao *et al*., 2008).

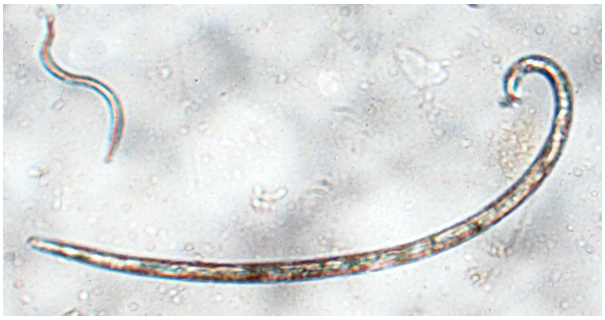

Figure 1 – Pinewood nematode, *B. xylophilus* strain 8A (O.M. 40X amplification).

Under natural conditions, the life cycle of PWN involves a propagative cycle and a dispersal cycle (Figure 2). The wounds made by maturation feeding of *Monochamus alternatus* on living twigs are the starting point of the infection, with dauer juveniles transported by the sawyer; the transmitted dauer juveniles invade the wood tissues immediately, molt to adults, begin to feed and reproduce in resin canals and on the epithelial cells (phytophagous phase). During winter and early spring, nematodes pass through a resting stage and survive unfavourable conditions, aggregating in the xylem tissue and surrounding the pupal cell of the pine sawyer. In late spring, coinciding with the time of pupation, aggregated nematodes molt to dauer juveniles. Immediately after emerging, the adult beetles' respiratory system becomes contaminated with nematodes, hence *B. xylophilus* is also attracted by CO_2 released during beetle respiration. (Linit, 1988). They can molt into adults within 48 hours after transmission to a conifer host (Ichinohe, 1988).

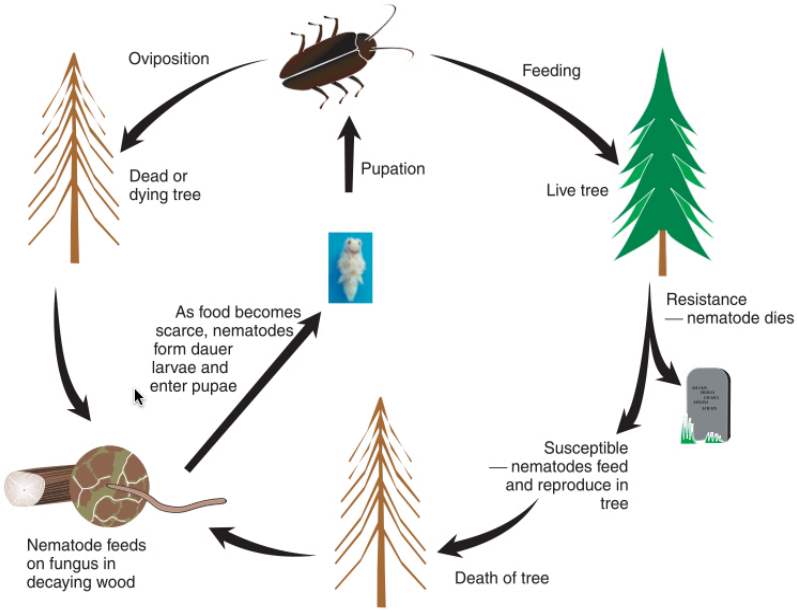

Figure 2 – Life cycle of *B. xylophilus* (from Jones *et al*. 2008).

As referred previously, *B. xylophilus* also has a fungi-feeding phase (mycophagous phase) that occurs in freshly cut softwood and in dead or dying conifers, usually resulting of secondary transmission during egg-laying by infested sawyer beetles. At this stage, the pinewood nematodes feed on fungi such as *Ceratocystis* spp., *Botrytis cinerea* and *Ophiostoma minus*, fungi that typically invade cut timber and dead or dying softwood (Oh *et al*., 2009).

Pine wilt typically kills pines older than 10 years within a few weeks to a few months and, despite all advances in research on this subject, the pathogenic mechanism of PWD has not been clearly defined. In its native country, the nematode isn't considered a pathogen, but when it spread throughout the world, massive damage occurred in forests, consequently damaging the economy of each zone. As the vector feeds on different types of plants, the disease attacks conifers such as pine trees (*Pinus* spp.), but also non-conifers, such as trees of the family Pinaceae, namely, *Picea*, *Abies*, *Cedrus* and *Larix* (Kobayashi *et al*., 1984).

In North America, the native country of *B. xylophilus*, the prevalent vector specie is *Monochamus carolinensis* (Zhao *et al*., 2007(a)); *M. alternatus* is the major vector in Japan (Kobayashi *et al*., 1984) and, in Europe, of the 150 different species of the genus *Monochamus* Dejean, only five endemic species occur, namely *M. galloprovincialis* (Olivier),

M. sartor (Fabricius), *M. sutor* (Linnaeus), *M. urussovi* (Fischer) and *M. saltuarius* (Gebler) (Naves *et al*., 2005; Naves *et al*., 2008).

In Portugal, the PWN is transported by *M. galloprovinciallis,* an endemic specie (Naves *et al*., 2007), and its primary host is *P. pinaster* Ait. (Mota *et al*., 1999) (also called maritime pine, or "pinheiro bravo"). The pine species *Pinus sylvestris* and *Pinus halepensis* are also favorable hosts for the PWN, but luckily their distribution and abundance is limited in Portugal (Mota and Vieira, 2008). Recent host experiments, under laboratory conditions, showed that Portuguese populations of *M. galloprovincialis* exhibit preferences for *P. sylvestris* among several pine species for nutrition (Koutroumpa *et al*., 2009). These alternative preferences can eventually redirect *B. xylophilus* to infest other type of trees and that's why more studies on this matter are required, since Portuguese flora is so diverse.

P. pinea (also called stone pine, or "pinheiro manso"), another important pine species in Portugal, is an interesting species given that, in nature, it isn't affected by the disease. It seems that the vector doesn't feed nor colonize this pine species, but the nematode itself can invade, multiply, infect and kill the tree (Zhao *et al*., 2008), but more slowly than in *P. pinaster* (Mota and Vieira, 2008).

Considering the risks of dispersal of this disease, strict regulations have been developed to eliminate the pest, particularly when importing wood chips, lumber and logs; procedures designed to disinfest transported coniferous wood include irradiation, fumigation, chemical dips and the use of elevated temperatures - 56 ºC for 30 min (Dwinell, 1997). After the nematode was found infecting Portuguese maritime pine, a national programme for the control of the PWN, "PROLUNP", was implemented. Within an area of 30 km radius, south-east of Lisbon, all symptomatic trees were clear-cut and a phytosanitary strip surrounding the affected zone was established (Jones *et al*., 2008). Some strategies for eliminating the pine wilt disease focus on the vector, by trying to eradicate the beetle or to depress its reproduction, but this doesn't ensure good results because of the high potential of pine sawyer reproduction and dispersal (Zhao *et al*., 2008).

PWN dispersion and multiplication are key factors in the occurrence of disease symptoms and tree death. (Ichihara *et al.*, 2000) The symptom development is the only tool that allows an observer to identify a diseased tree so this information has been distributed

throughout the forest authorities. During the different stages of the disease (from the early one to the advanced) the symptoms aggravate, ultimately resulting in host death. Between the main symptoms we find cessation of oleoresin exudation from the stems, decrease in photosynthesis, denaturation of xylem and cortex parenchyma cells, traumatic resin canal formation, cambium destruction, production of phytotoxic substances, enhanced respiration and ethylene production (Fukuda, 1997). These traumas are caused by nematode movement through resin canals, rays and cambial zone, which play an important role in transportation or storage of assimilated materials that are indispensable for maintaining the life of a tree (Kusunoki, 1987). Mamiya (2008) also reported that they might use tracheids as a passage for movement. However, the most visible symptom of all is wilting of leaves. This is a result of the other symptoms referred above, appearing in the late stage of the disease (Fukuda, 1997). The needles initially turn greyish green, then tan-colored and finally brown. The entire tree turns brown all at once, which complicates the early detection of the disease (Gleason *et al*., 2000).

The virulence of the nematode to the tree varies widely and some avirulent strains have been described in nature that when pre-inoculated induce resistance in pine to a post-inoculation with virulent nematodes (Kosaka *et al*., 2001). This induced resistance, comparable with immunity in animals, has been studied as an alternative control method for the disease, although the mechanism remains to be solved (Takeuchi *et al*., 2006). Whether involving avirulent strains, nematicides or chemical formulations, the process of preventive "vaccination" is being studied in order to prevent the reproduction or to kill PWN within the tree; this route for disease control is very "environment friendly" since it doesn't have any negative consequence and, on the other hand, remains effective for several years; formulations like *Morantel tartrate*, *Levamisol hydrochloride*, *Mesulfenfos* and *Nemadectin* are already registered for use (Zhao *et al*., 2008). However, these chemical agents are of limited value due to poor water solubility, lack of therapeutic efficacy and/or high cost (Oh *et al*., 2009).

As for other virulence mechanisms, the presence of bacteria on the nematode surface has been described and has been shown that *Pseudomonas* and *Pantoea* were present in trees infected with *B. xylophilus* but absent from uninfected trees (Jones *et al*., 2008). Also, Kwon *et al*. (2010) isolated *Burkholderia arboris*, *Brevibacterium frigoritolerans*, *Enterobacter asburiae*, *Serratia marcescens* and *Ewingella americana* from *B. xylophilus* and showed in

their studies that antibacterial agents had a suppressive effect on pine wilt disease, which may indicate that bacteria have an important role in nematode pathogenicity. It was shown that some of the toxins responsible for the disease could not be produced by the nematode, leading to the conclusion that the bacterial population attached to the surface of PWN were responsible for that; also supporting this hypothesis are studies which revealed that the inoculation of aseptic nematodes didn't lead to visible symptoms development (Zhao *et al*., 2008).

The selection of resistant pine trees from natural forests or plantations is another method for preventing the disease. As the need for resistant pines increases, the gathering of resistance genes in a single cultivar is being explored by crossing resistant clones, which would make resistance higher. In what concerns selection breeding, it's important that the selected trees don't attract the vector and/or that they can tolerate the pathogen, so usually surviving trees from nematode damaged areas are selected; it's also possible to create new varieties with specific characteristics of interest by repeated crossing and backcrossing (Zhao *et al*., 2008).

The number of nematodes that enter a healthy tree is directly proportional to the number of nematodes carried by the vector (Maehara *et al*., 2005) and an important factor that affects the number of nematodes carried by beetles, is the fungal flora in the wood; for example, when *Trichoderma* spp. or *Verticillium* sp. is prevalent in the host, the number of nematodes decrease (Maehara, 2008). These are endoparasites that stop nematode reproduction and kill nematodes, respectively (Zhao *et al*., 2008). Other fungi were reported to have nematicidal activity, such as *Paecilomyces* spp. (Liu *et al*., 2009) or *Fusarium bulbicola*, that produce beauvericin, a nematicidal compound (Shimada *et al*., 2010).

Monoterpenes are a group of volatile plant secondary metabolism compounds that play an important role in mediating interactions between the plant and its environment and can induce defence responses that enable plants to differentiate between insect damage and mechanical wounding (Mateus *et al*., 2010). Certain volatile extracts are commonly activated in the biosynthetic pathways of a wide range of plants, namely α-pinene (which is estimated to achieve about 85% of Pinaceae essential oil constituents), β-pinene, limonene and myrcene (Proença da Cunha, 2005) and, when infected, these trees emit a characteristic assembly of volatile compounds that attract *Monochamus* species to them. Also, plant essential oils are

being studied for their potential as control agents against the disease as they have nematicidal activity against PWN (Kim *et al.*, 2008). On the other hand, chemotaxis has an influence in host location by nematodes (Zhao *et al*., 2007(b)).

Gas Chromatography – Mass Spectrometry (GC-MS) is the most frequently used method for the identification of volatiles, but its applicability is still arguable, since tree-emitted volatiles are very complex, complicating the total separation of all components (Mateus *et al*., 2010). Moreover, in most studies, the volatiles are being isolated by simultaneous distillation-extraction (SDE), which includes the extraction solvent that frequently co-elutes with less retained peaks during chromatographic run (Santos *et al*., 2006). The alternative method to surpass this problem is headspace solid-phase microextraction (HS-SPME) that dismisses solvent intervention (Supleco, Bulletin 923A).

According to Rutherford *et al*. (1992), *B. xylophilus* moves faster at higher temperatures, and that's why symptoms develop drastically during the summer. In this time of the year, trees suffer from water stress, and transpiration in pine leaves decrease, which lowers water potential. The decrease in stem and leaf water content has been studied and usually occurs shortly before visible symptoms appear in nematode inoculated plants (Tan *et al*., 2005). Therefore, water flow and retention may be a major contributing factor for disease pathology in pine wilt (Bolla *et al*., 1986) and high temperature stress is also a major abiotic factor that accelerates pine tree death, as it promotes *B. xylophilus* multiplication (Sikora and Malek, 1991).

As the invasion by nematodes start, it is thought that an innate hypersensitive defense mechanism is triggered. This hypersensitive reaction results in the release of phenolics, synthesis of toxins and phytoalexins and the compartmentalization of xylem and other tissues, followed by flooding of tracheids with oleoresin and toxic substances (Myers, 1988). Recent studies point out that these basal defence mechanisms against pathogens occur within the first couple of hours after infection (Baldo *et al*., 2010) and that HR is activated by a genetic program, where resistance genes recognize certain effectors thus initiating a resistance response that is frequently linked to a rapid cell death (Schiffer *et al*., 1997). As studies with cordycepin treatment (an inhibitor of mRNA synthesis) suggest that HR preventing is more successful if performed within the first 4 hours, one can infer that mRNAs that are required for the HR are synthesized in the host prior to 4 hours after inoculation (Hein *et al*., 2004).

Nowadays, studies are addressing potential molecular targets for anti-nematodal drugs. For example, Choi *et al*. (2010) demonstrated that the cdc-25 gene is common to female and male *B. xylophilus* with an important role in their cell cycle regulation mechanism. This gene could, therefore, be a potential target for an RNAi silencing mechanism. There is also a pressing necessity of new methods of PWN detection that allow quicker diagnosis of infected trees and prevent its introduction in pest free areas; consequently, some authors have been developing real-time PCR assays using DNA topoisomerase I gene of *B. xylophilus* as a target (Huang, L. *et al*., 2010). Also, studies have been directed to contribute to the disclosure on molecular regulation and evolution of miRNA in plant parasitic nematodes (Huang, Q-X. *et al*., 2010).

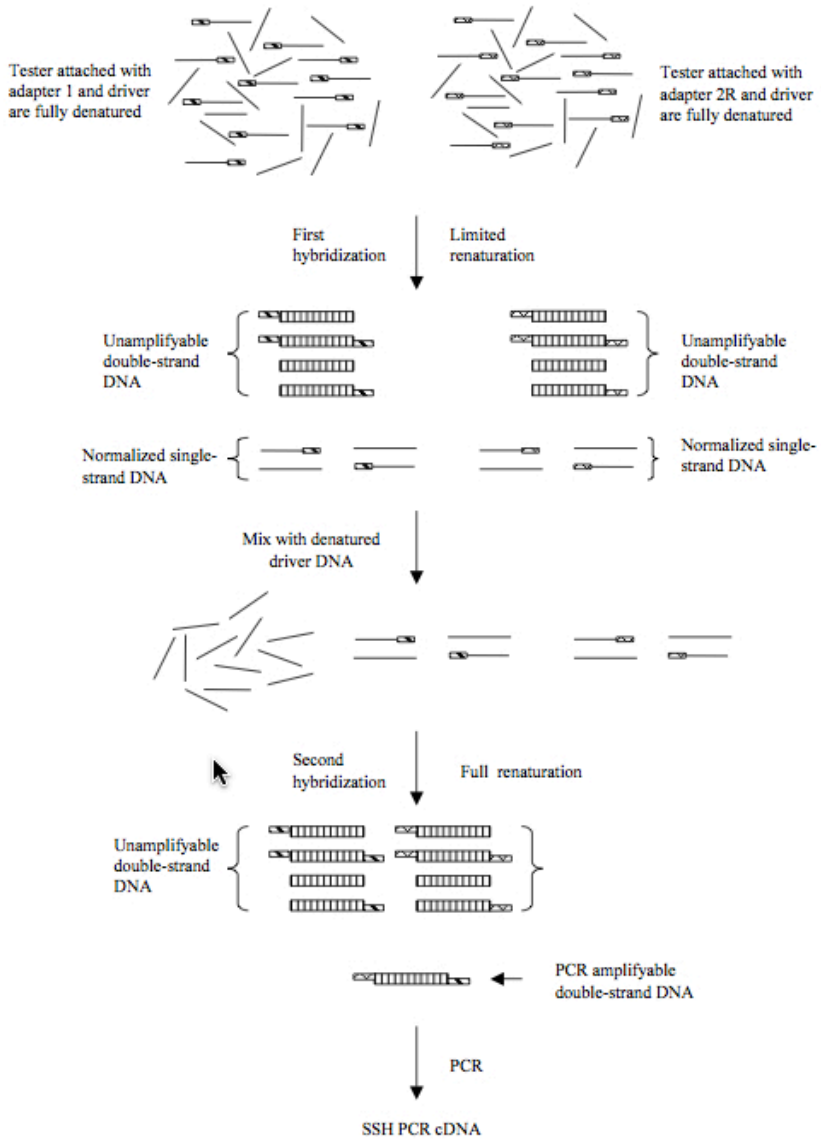

Figure 3 – Scheme of the SSH method (from Ji *et al*., 2002).

Sadly, not much is known about the pine genome and more is needed to unveil about this disease at a molecular level. Analysis of expressed sequence tags (ESTs) by single pass random sequencing of cDNA libraries is a powerful tool for rapid and cost-effective gene discovery (Kikuchi *et al*., 2007). Diatchenko *et al*. (1996) described the method of suppression subtractive hybridization (SSH) as a useful technique to identify and isolate cDNA fragments differentially expressed, based on suppression PCR effect where long inverted terminal repeats, when attached to DNA fragments, can selectively suppress amplification of undesirable sequences in PCR procedures (Figure 3); plus, this method incorporates a hybridization step that normalizes sequence abundance during the course of subtraction, enriching only the differentially expressed cDNAs. Built on these principles, SSH allows one to hybridize cDNA from one population (tester) to excess of cDNA from another population (driver), to then enrich the unhybridized fraction (target) and reject the hybridized common sequences (Figure 3).

Shin *et al.* (2009) constructed SSH libraries and assessed their functions in relation to the physiological responses of PWN in 8-year-old Japanese red pines, *P. densiflora*, 21 hours after infection (hai) and 1 and 7 days after infection (dai). Also, loblolly pine, *P. taeda*, has been studied for its response to water stress (Lorenz *et al.,* 2006) using single-pass DNA sequencing. Other molecular analyses and symptom development during PWD have been studied in *P. thunbergii* (Tan *et al*., 2005), *P. radiata* (Li *et al.*, 2010) and *P. sylvestris* (Palmé *et al.*, 2008). The most studied species are Japanese ones, since this is one of the most affected areas by the nematode.

In this work we report on the utilization of SSH technique for identification of genes differentially expressed by *P. pinaster* and *P. pinea* after being infected with pine wood nematode. Also, as referred above, it's thought that an early stage of the infection may trigger an immediate genetic response, which led us to focus on the first hours after pinewood invasion. This will give us hints on genes that may be involved in the resistance mechanism to the PWD to further enrich our knowledge on the behaviour of these two species that have dissimilar susceptibility to the disease. We also report on the phenotypical and metabolic differences between infected and non-infected *P. pinea* and *P. pinaster* trees, at an early infection stage.

More than 30 years have passed since *B. xylophilus* was found to be the causal agent of PWD (Kikuchi *et al*., 2007). Hopefully, this study will contribute to a better understanding of some of the molecular, physiological and metabolic aspects of pine wilt disease, in particular in two very important pine species to the Portuguese forestry sector.

2. OBJECTIVES

General objectives

Given the small amount of available information regarding the pathogenesis of *B. xylophilus* in the Portuguese pine populations, the general objective of this work was to better understand pine wilt disease mechanisms and how this disease affects *P. pinaster* and *P. pinea* at an early stage of infection, at a physiological and molecular level.

Specific objectives

To inoculate young trees of *P. pinaster* and *P. pinea,* two pine species with different susceptibilities to the PWN, with a virulent geographical isolate of *B. xylophilus*, and, at 3 hai,

- Analyse the effect of nematode inoculation in the chlorophyll concentration of the needles;
- Compare the effect of nematode inoculation on the dehydration of the stems;
- Conduct an exploratory analysis of total volatile production between *P. pinea* and *P. pinaster*, using GC technique.
- Generate a cDNA library of ESTs from *P. pinaster* (more susceptible pine) and *P. pinea* (less susceptible pine).
- Implementation of the SSH technique for the study of pine differentially expressed genes.
- Cloning of differentially expressed genes between *P. pinea* and *P. pinaster*-inoculated plants using the SSH technique.

3. MATERIALS AND METHODS

Plant material

The plant material utilised in these studies were *Pinus pinea* and *Pinus pinaster*, approximately two years old, from Viveiro Anadiplanta (Anadia, Aveiro; 40º26'27" N, 8º25'47" W). The pine trees were transferred from the vials to vessels with equal parts of black peat (Siro Plant, Vegetal Substract) and exfoliated vermiculite. Pine trees were grown in controlled growth chamber conditions, in an Aralab Fitoclima 10000EHF, with relative humidity of 80%, and plants were exposed to a 16h day / 8h night photoperiod at about 490µM. The temperature was kept at 24-26 ºC during the light period and at 19-20 ºC during the dark period.

Nematode culture

Square pieces with about 1cm^2 of Potato Dextrose Agar with *Botrytis cinerea*, grown at 26ºC for 7 days, were transferred to test tubes with barley grains Seara® previously autoclaved. These tubes were incubated at the same conditions mentioned above. Then, small pieces with pre-grown *B. xylophilus* geographical isolate HF (isolated from Setubal Region), were put inside the test tubes and incubated at the same conditions again. The multiplied nematodes were extracted using Baermann funnel technique (Baermann, 1917) prior to inoculation. Only nematodes that that had been extracted for less than 2 hours were used in the subsequent experiments.

Inoculation and sampling time

Following Futai and Furuno (1979) method, a suspension with 1000 nematodes was pipetted on a small wound made with a scalpel on the main stem with 3-5 cm, about 40 cm above ground level. The inoculated wounds were covered with parafilm to prevent drying of the inoculums.

Three hours after inoculation, for each experimental sample, the entire pine tree stem was cut in small pieces and stored at -80 ºC until further analysis.

Physiological changes

Four different treatments were made for the following protocols, using the same inoculation conditions mentioned previously. Thirty *P. pinaster* and 30 *P. pinea* trees were

randomly selected and inoculated with *B. xylophilus* strain HF (30 control pine trees of each species were inoculated with sterile water).

Determination of water content

Sample stems were lyophilised for 72 h; water content and decrease of water content were calculated as demonstrated by Tan *et al*. (2005) from the equations: water content (%) = (sample fresh weight – sample dry weight) x 100 / sample fresh weight; and decrease of water content (%) = (water content of control plant - water content of inoculated plant) / water content of control plant x 100].

Total chlorophyll extraction and quantification

The protocol followed was the one of Abadía *et al*. (1984), with slight modifications. In short, 12,5ml of 4g/L solution of $CaCO_3$ in methanol were added to 0,5g of pine needles. After an incubation period of 48 hours in the dark, at room temperature, the material was white due to the loss of the pigments to the methanolic solution. One ml of the supernatant was diluted in 25 ml of deionized water and absorbance measures were taken at 663 nm and 645 nm. Spectrophotometric chlorophyll quantification of total chlorophylls were calculated following the equation:

$$(8{,}02 \cdot A_{663} + 20{,}21 \cdot A_{645}) \frac{0.0125 \text{ x Dilution Factor}}{\text{Fresh weight (g)}}$$

Gas chromatography analysis

Four different treatments were carried out for this method, using the same set up described initially in this chapter. Seven plants of *P. pinaster* and 7 of *P. pinea* were inoculated with nematode HF, and 7 trees of each species were inoculated with deionized water to be used as control.

The analysis of the volatile compounds was performed by gas chromatography, using a HP 5890A gas chromatograph (Hewlett Packard, USA) equipped with flame ionization detection (GC-FID). The separation was achieved on a Stabilwax-DA column with 60m, 0.25mmID, 0.25µm (RESTEK) after split splitless injection, using hydrogen as carrier gas, at a flow rate of 30 ml/min. Oven temperature was initially held at 40ºC for 1 min and increased up to 220ºC, at a rate of 2ºC/min where it was held for 10 min. The flame ionization detector temperature was set at 220ºC.

Extraction of total RNA

Eight pine trees arbitrarily selected were used: four pooled *P. pinaster* trees (tester) and four pooled *P. pinea* trees (driver), both inoculated with *B. xylophilus* strain HF. Tester and driver total RNA was extracted separately and the extraction was performed according to an optimized method from Provost *et al*. (2007). The following solutions were prepared in advance: extraction buffer (2% CTAB; 2% PVP; 100mM Tris-HCl pH 8.0; 25mM EDTA; 2.0M NaCl; 0.5g/L spermidine); chloroform-isoamyl-alcohol (24:1); 10M LiCl; 5M NaCl.

Approximately 200 mg of sample was ground with liquid nitrogen until a fine powder was obtained, and placed in an eppendorf tube containing 1mL of Extraction Buffer with 2% of β-mercaptoethanol. Then the mixture was vortexed and incubated for 10 min at 65 ºC (inverting the tubes every 2-3 min). One mL of CIA was added and the solution was mixed and centrifuged at 9,500*xg* for 10 min at room temperature. The supernatant was transferred to a sterile tube and 800µl of CIA was added. The mixture was vortexed and centrifuged at the same conditions above. A total volume of 125µl of LiCl was added; the solution was mixed and incubated o.n. at 4 ºC.

The following morning, the mixture was centrifuged at 9,000*xg* for 20 min at 4 ºC and the supernatant was discarded. After the pellet was dried out by inverting the tube, it was resuspended with 500µl of STE buffer (TRIS NaCl EDTA buffer, pH 7.8) and 450µl of CIA were added. The mixture was centrifuged at 9,500*xg* for 15 min at 4 ºC and the supernatant transferred to a clean tube. The solution was kept on ice and 150µl of STE was added to the previous tube, to extract the remaining RNA. 100µl of NaCl and 1,5mL of cold absolute ethanol were added and the solution was incubated 30 min at -80 ºC. The mixture was centrifuged at 9,500*xg* for 20 min at 4 ºC and the supernatant was discarded. The pellet was resuspended in 400µl of cold 70% ethanol and then centrifuged at 9,500rpm for 10 min at 4 ºC. The supernatant was discarded and the pellet was dried out by inverting the tube.

The pellets were resuspended in 30µl of DEPC-Water (Sigma) and stored at -20 ºC. RNA quality and quantity were checked with UV-spectrophotometry, using a nanophotometer (Implen, Isaza, Portugal).

mRNA synthesis

Oligotex mRNA Midi kit (Qiagen, Cat. Nr. 70042) was used to synthesize mRNA from total RNA previously extracted. To 250 µl, with about 0,25 mg of total RNA, 250 µl of supplied OBB buffer and 15 µl of previously 37 ºC heated Oligotex Suspension were added. The mixture was heated to 70 ºC for 3 min and incubated at 25 ºC for 10 min. A

centrifugation at 14,000*xg* for 2 min was performed and supernatant discarded. The pellet was resuspended in 400 µl of OW2 Buffer, loaded into a spin column and centrifuged for 1 min at 14,000*xg*. The flow-through liquid was discarded and spin column membrane was washed again with OW2 Buffer; a new centrifugation was performed, in the conditions indicated above. Spin column was transferred to a new eppendorf and, after pre-heating OEB Buffer to 70 ºC, 20 µl of this were added to the column and homogenised with the membrane by pipetting up and down. The mRNA was eluted with a 1 min centrifugation at 14,000xg. To ensure maximal yield, a new elution was performed, passing the eluate through the membrane again and submitting the tubes to a new centrifugation.

cDNA Synthesis

Tester and driver cDNA were synthesized using SMARTer^{TM} PCR cDNA Synthesis Kit (Clontech Laboratories, USA, Cat. Nr. 634925), according to manufacturers' instructions. Briefly, tester and driver first cDNA strand was synthesized by adding to 3.5 µl of RNA, 1 µl of 3' SMART CDS Primer II A. This mixture was incubated in a hot lid thermal cycler at 72 ºC for 3 min and 42 ºC for 2 min. A master mix was prepared with 5X First-Strand Buffer, DTT (100 mM), dNTP Mix (10 mM), SMARTer II A Oligonucleotide (12 µM), RNase Inhibitor and SMARTScribe Reverse Transcriptase (100 U), which was added to the mixture and then incubated at 42 ºC for 1 hour and 70 ºC for 10 min.

The first-strand reaction product was diluted by adding 40 µl of TE buffer and cDNA amplification was performed by LD PCR. Thermal cycler was preheated to 95 ºC and a master mix (containing deionised H_2O, 10X Advantage 2 PCR Buffer, 50X dNTP Mix (10 mM), 5' PCR Primer II A (12 µM) and 50X Advantage 2 Polymerase Mix) was added to 30 µl of the previously obtained cDNA.

Part of the resulting PCR reaction mix was then submitted to an optimization thermal cycling and following conditions were selected: 95 ºC for 1 min; 21 cycles with 95 ºC for 15 sec, 65 ºC for 30 sec and 68 ºC for 3 min. Two µl of 0.5M EDTA was added to each tube to terminate the reaction.

Two different methods for product purification were tested. In the first method, column chromatography was performed, by adding phenol:chloroform:isoamyl alcohol (25:24:1) to the product and centrifuging at 14,000 rpm for 10 min. The aqueous layer was placed in a clean tube and 700 µl of n-butanol were added. The mixture was centrifuged at 14,000 rpm for 1 min at room temperature, and the upper phase discarded. After preparation of the chromatography column, the sample was applied to the center of the gel bed's flat

surface, and washes with 1XTNE buffer were made. An agarose/SybrSafe gel analysis was performed, to confirm the presence of PCR product in the purified ds cDNA fraction.

The second method used to purify the product was illustra GFX PCR DNA Purification kit (GE Healthcare, Cat. Nr. 28-9034-71). A capture buffer was added to 100 µl of sample and when the pH indicator was yellow, the mixture was transferred to a previously prepared column and centrifuged for 30 sec at 16000*xg*. Flow-through liquid was discarded and wash buffer added to the column, which was again centrifuged with the same conditions mentioned above. A final volume of 10 µl of Elution Buffer was added to the membrane and, after an incubation period of 10 min at room temperature, a final centrifugation was made at 16000*xg* for 10 min. A second elution was done in this final purification step.

The purified ds cDNAs obtained by the second method were then digested with RsaI enzyme, with an o.n. incubation at 37 ºC, and purified again with illustra GFX PCR DNA Purification kit (GE Healthcare, Cat. Nr. 28-9034-71).

The yield of DNA was assessed by UV spectrophotometry.

SSH Library Construction

SSH was carried out using PCR-Select^{TM} cDNA Subtraction Kit (Clontech Laboratories, USA, Cat. Nr. 637401). Forward and reverse subtractions were performed. In short, tester, driver and skeletal muscle tester cDNAs were ligated to adaptors by adding to the sample a master mix (containing sterile water, 5X ligation buffer and T4 DNA Ligase (400units/µl)) and adaptors 1 and 2R (10 µM each), which sequences are presented in Table 1. After an o.n. incubation period at 16 ºC, EDTA/Glycogen mix was added to terminate the reaction and the mixture was incubated at 72 ºC for 5 min.

Table 1 – Adaptor 1 and 2R sequences used in SSH protocol

Adaptor	***Sequence***
1	5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3'
2R	5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3'

For the forward hybridization a master mix was prepared with the driver cDNA, the adaptor 1 ligated tester cDNA, the adaptor 2R ligated tester cDNA and 4X Hybridization buffer (the same conditions were kept for the reverse subtraction, using driver cDNA as tester). After a brief centrifugation, the mixture was incubated at 98 ºC for 1.5 min and 68 ºC for 8 hours. Second hybridization requires freshly denaturated driver cDNA, which was added

to the first hybridization product. The mixture was incubated o.n. at 68 ºC and diluted in dilution buffer. After incubation at 68 ºC for 7 min, the sample was ready for PCR amplification.

One µl of diluted cDNA was aliquoted into a tube and a Master Mix was prepared with sterile water, 10X PCR reaction buffer, dNTP Mix (10mM), PCR Primer 1 (10 mM) and 50X Advantage cDNA Polymerase Mix. The mixture was vortexed, centrifuged and incubated at 75 ºC for 5 min after which, immediately started thermal cycling with the following conditions: 94 ºC for 25 sec; 27 cycles with 94 ºC for 10 sec, 66 ºC for 30 sec and 72 ºC for 90 sec. Again, a Master Mix was prepared with sterile water, 10X PCR reaction buffer, nested PCR primer 1 (10 µM), nested PCR primer 2R (10 µM), dNTP Mix (10 mM) and 50X Advantage cDNA Polymerase Mix (primer sequences used in this protocol are presented in Table 2). After vortexing and briefly centrifuging, the master mix was added to the reaction and overlaid with one drop of mineral oil. Thermal cycling was immediately started with the following conditions: 12 cycles with 94 ºC for 10 sec, 68 ºC for 30 sec and 72 ºC for 90 sec. The resulting product was stored at -20 ºC for further analysis.

Table 2 – Adaptor 1 and 2R sequences used in SSH protocol

Primer	***Sequence***
PCR Primer 1	5'–CTAATACGACTCACTATAGGGC-3'
Nested PCR Primer 1	5'-TCGAGCGGCCGCCCGGGCAGGT-3'
Nested PCR Primer 2R	5'-AGCGTGGTCGCGGCCGAGGT-3'

Cloning

Subtracted and unsubtracted cDNAs were cloned into the pCR2.1-TOPO Vector (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA, USA), accordingly to manufacturers' instructions. To do so, fresh PCR product was mixed with salt solution and TOPO Vector and incubated at room temperature for 30 min. Prior to *E. coli* transformation, S.O.C. medium was warmed to room temperature and selective plates (LB plates containing 50 µg/ml ampicillin) to 37 ºC and competent cells were thawed on ice. Two µl of cloning reaction were added to a vial of One Shot Chemically Competent *E. coli* (TOP10) and mixed gently. After 30 min incubation on ice, the cells were heat-shocked for 30 sec at 42 ºC. The tubes were immediately transferred to ice, S.O.C medium was added and incubated horizontally with agitation (200 rpm) at 37 ºC for 1 hour. Ten and 50 µl from each transformation were spread on a selective plate containing 40 µl of 40 mg/ml solution of X-gal (Sigma Cat. Nr. B4252)

and 50 μg/ml of ampicillin and incubated o.n. at 37 ºC. White colonies were selected and grown to saturation in LB Broth with ampicillin (50 μg/ml) at 37 ºC with agitation (230 rpm) for plasmid DNA extraction.

Plasmid DNA Extraction

Plasmid DNA Extraction was performed with GenElute Plasmid Miniprep kit (Sigma, Cat. Nr. PLN70) according to manufacturer's instructions. Shortly, *E. coli* cells previously used to clone the cDNA fragments were centrifuged and the pellet was resuspended in Resuspension Solution. Lysis Solution was added and incubated for 5 min. Neutralization Solution was added to the lysate and this mixture was transferred to a previously prepared binding column. The resuspension, lysis and neutralization solutions were provided by the kit. The flow-through liquid was discarded and treated with Wash Solution. The column was transferred to a clean tube and 75 μl of sterile water was added. After a 12,000*xg* centrifugation for 1 min the product was stored at -20 ºC. In total, 90 samples were sent for sequencing to Macrogen (Korea).

Statistical Analysis

Relative area percentage of the elements present in the volatile fraction of GC analysis was used for data analysis. Data processing was performed using PASW Statistics 18 statistical package. Principal component analysis was used to determine pine species homogeneity and treatment separation.

All other analysis and graphics, concerning chlorophyll extraction and water content were obtained using Microsoft Excel and GraphPad Software.

4. RESULTS AND DISCUSSION

Sequencing of ESTs from cDNA libraries is one of the latest methods to acquire information on a transcriptome of interest. These ESTs are short and partial sequences of a transcribed nucleotide sequence produced by sequencing of a cloned mRNA from cDNA libraries, and represent portions of expressed genes. The most highly expressed genes will be represented many times by identical or nearly identical clones in the libraries (Li and Brouwer, 2009).

Lorenz *et al.* (2006) explored data from single-pass DNA sequencing of a collection of 12,918 cDNAs from the root tissues of loblolly pine, *P. taeda*, to obtain further insight into the conifer response to water stress. Dubos and Plomion (2003) studied this problem in *P. pinaster* roots, which provided functional and expressional candidate genes that can serve as molecular markers for identifying quantitative trait loci (QTL).

Shin *et al.* (2009) isolated 2778 ESTs from two SSH libraries and assessed their functions in relation to the physiological responses of PWN-infected trees. Their plant material was 8-year-old Japanese red pines, *P. densiflora*, from a PWN-damaged area and also 4-year-old trees grown in nursery for 3 years. The time points studied by this group were 21 h after inoculation and 1 and 7 days after inoculation. The authors isolated and characterized upregulated or newly induced genes related to biochemical and physiological responses of pine trees as a result of PWN invasion.

In what concerns the study of disease development, Tan *et al*. (2005) evaluated external symptoms (amount of resin exudation, drying and pith colour) in the stem of 3-4 year old Japanese black pines, *P. thunbergii*, and determined sample water content through a time-course study with time points of 5, 10, 15, 20 and 70 days after inoculation. They concluded that symptoms developed drastically 15 days after inoculation and that stem water content diminished from 50% to 30% between 15 and 70 days after inoculation, ending up in tree death.

Other trees have been studied, but mostly Japanese ones. For example, Japanese larch, *Larix kaepferi*, has been studied for its susceptibility to the nematode. Mamiya and Shoji (2009) compared symptom development in larch tree with *P. thunbergii* since this one is

known for its susceptibility. Of the 45 nematode inoculated seedlings of 2-year-old *L. kaempferi*, 98% succumbed to the disease. This indicates that there is a need to study possible hosts for this disease hence it is devastating for its susceptible plants.

In this report, *P. pinaster* and *P. pinea,* the most abundant pine populations in Portugal, were the selected tree hosts for the study of PWD, 3 hours after infection with PWN. In this chapter results of SSH library construction, chlorophyll concentration, water content and volatile compound production will be presented.

4.1. *Physiological changes*

Wilting of leaves is the most critical symptom in PWD and it is believed to be a result of decrease of transpiration and water potential; also it is regarded as a change in the plant at an advanced stage of infection (Fukuda, 1997). This study concerns the early stage of PWD, at the first 3 hai.

Water content results

Nematode infection causes blockage of water conduction and reduced transpiration (Tan *et al.*, 2005). Three hours after nematode invasion, the water content of the stem of both *P. pinaster* or *P. pinea* decreased in comparison with control trees (Figure 4).

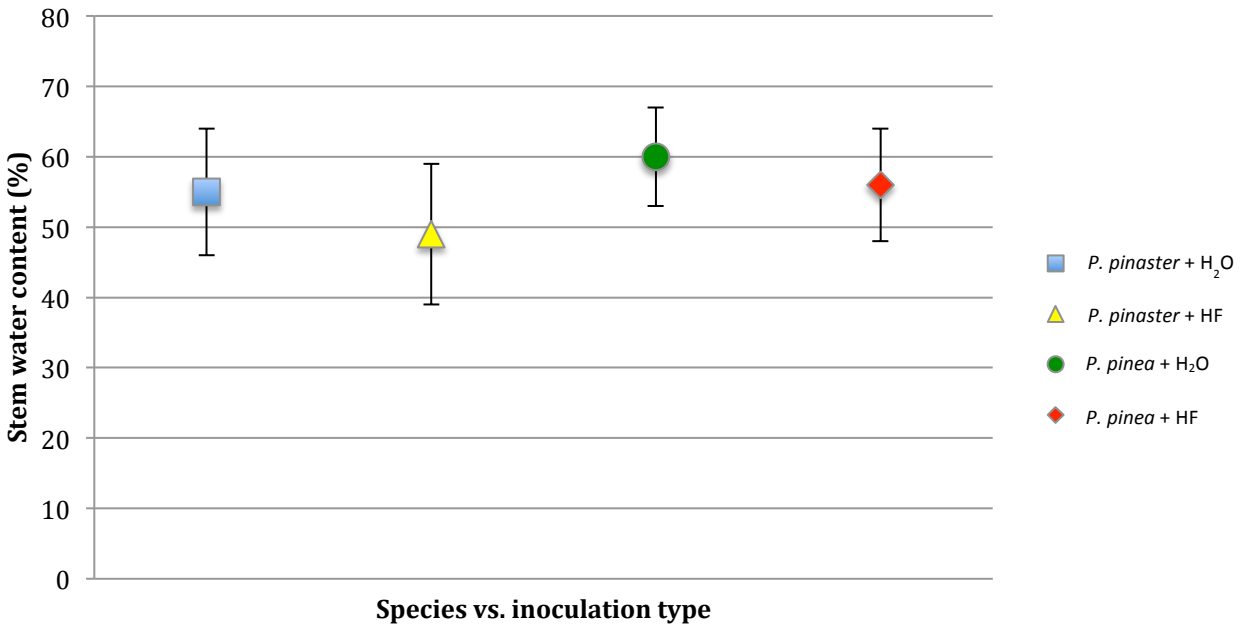

Figure 4 – Stem water content of *P. pinaster* inoculated with water (■) or *B. xylophilus* strain HF (△) and in *P. pinea* inoculated with water (●) or *B. xylophilus* strain HF (◆). Each value respresents a mean of 30 biological replicates; bar is SD.

The results showed a significant decrease of water content due to nematode inoculation of 10,9% in *P. pinaster* (p<0.05) and 6,7% in *P. pinea* (p>0.05), but statistical analysis only supported the difference between *P. pinaster* control and nematode infected. Not much is known about *P. pinea* as a host of PWD and why this species appears to have

lower susceptibility to the nematode (Mota and Vieira, 2008), but our results show a clear difference between *P. pinaster* and *P. pinea* response to nematode infection ($p<0.05$) which may indicate that, in what concerns water content in stems, stone pine is less affected than maritime pine, the primary host of the disease in Portugal. This might suggest, as noted by Futai (2003), that resistance in certain trees, like *P. taeda*, could be partly explained by the hydrophilc reppelants contained in it's bark, hence *Bursaphelenchus* species aggregate to the hydrophobic host substance(s), while their invasion of host tissues is controlled by hydrophilic host substance(s).

The development of wilt symptoms at high temperatures depends on the degree of nonreversible tracheid cavitation caused by nematode damage (Rutherford *et al*., 1992). The studies of Tan *et al.* (2005) on 3-4 years old Japanese black pines were conducted through 5 to 70 dai and registered a percentage decrease of 7.2% and 36.1%, 10 and 70 dai, repectively. Previous studies were conducted in 20-30 year old *P. sylvestris* and *P. strobus* (Bolla *et al*., 1986) during 3 months, and water content was evauated. The authors concluded that changes in water content of infected seedlings could result from physical or chemical blockage of the water transport system.

For an early stage of the disease this is an acute decrease in water content and indicates a strong and immediate response to the infection. Methods for early detection of PWD are urgent, so that the spread from tree to tree can be prevented. As shown in Figure 4, at 3 hai levels of water content in *P. pinaster* diminish, which can be a possible early indicator for an infected tree.

Chlorophyll extraction

Chlorosis in old leaves is a distinctive symptom of the advanced stage of the disease (Fukuda, 1997). Following, the results of chlorophyll concentration in *P. pinaster* and *P. pinea*, control and nematode-inoculated trees, are presented (Figure 5).

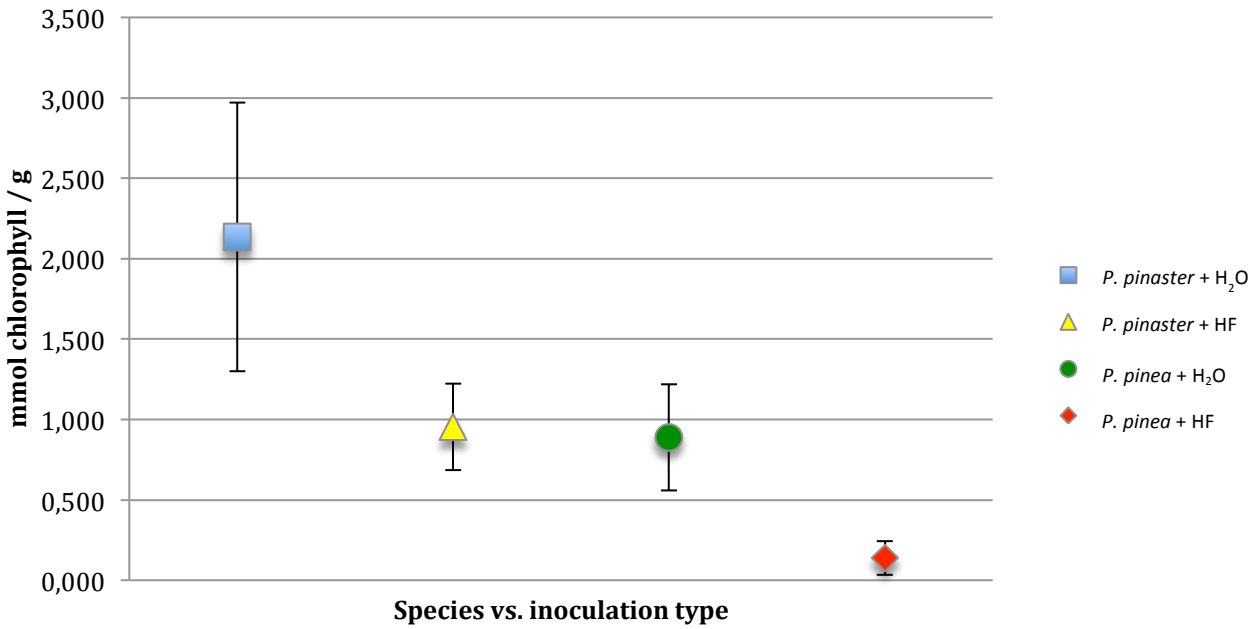

Figure 5 – Total chlorophyll concentration of *P. pinaster* inoculated with water (■) or *B. xylophilus* strain HF (△) and in *P. pinea* inoculated with water (●) or *B. xylophilus* strain HF (◆). Each value represents a mean of 30 biological replicates; readings were made in triplicate; bar is SD.

There is a natural color variation in the needles of *P. pinea* and *P. pinaster*, with *P. pinaster* having a darker shade of green. This is in accordance with the higher amount of total chlorophylls in the needles of this species, when compared with *P. pinea* ($p<0.0001$).

Moreover, after infection, the chlorophyll concentration decreased in both species, specially in *P. pinaster*. At naked eye both treatments seemed similar and no difference in needle coloration was detected. However, as shown in Figure 5, after the extraction method there was a significant difference in chlorophyll levels between water and nematode inoculated, that indicates that as early as 3 hai the chlorophyll levels of the needles quickly start to diminish, in a more abrupt way in *P. pinaster*. These differences between treatments in

chlorophyll concentration were statisticaly supported (p<0.0001) and show that the obtained values were not just a result of the mechanically made wound from the inoculation process. Also, differences between species response were extremely different in nematode-inoculated trees (p<0.0001).

Therefore, these results indicate that the diminishing in chlorophyll concentrations in the needles starts at an early stage of the disease, although needles only loose their green colour at a later stage of infection. Despite needle chlorosis being considered characteristic of an advanced phase of PWD, here we show that, although it is not visible to the naked eye, chlorophyll levels decrease rapidly in the first hours after infection which influences photosynthetic processes, leading to tree death.

4.2. *Gas chromatography – secondary metabolism analysis*

Plant volatiles may be constitutively emitted by the plants or induced under certain environmental conditions, and can play an important role in mediating interactions between plant and insect herbivores (Mateus *et al*., 2010). In order to compare the volatile patterns of *P. pinaster* and *P. pinea* inoculated with water or nematode, GC analysis was performed and, at 3 hai, a principal component analysis (PCA) was conducted. The resulting model had two significant principal factors, explaining 44,9% of the variance in the data (Figure 6).

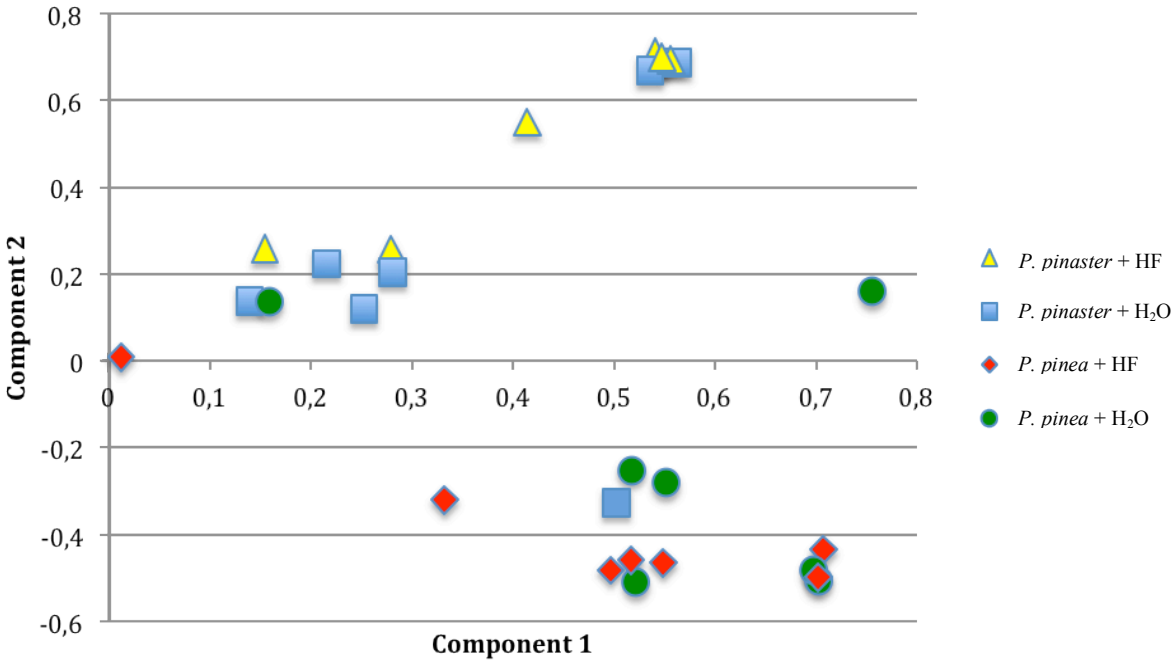

Figure 6 – Analysis of the volatile pattern of *P. pinaster* inoculated with water (■) or *B. xylophilus* strain HF (△) and in *P. pinea* inoculated with water (●) or *B. xylophilus* strain HF (◆). A total of 44,9% of the variance in the data is explained by the two significant principal components (24,6% of the variance of the **X** matrix and 20,3% of the variance of the variable **Y)**, as judged by cross-validation.

Two groups were fairly discriminated – nearly all *P. pinaster* samples were separated from *P. pinea* by the discriminant factor explaining 20,3% of the variance. The first discriminant factor, explaining 24,6% of the variance, didn't show any pronounced tendency in treatment (control *versus* nematode inoculated).

Santos *et al*. (2006) performed GC-MS analyses in order to see if a clear distinction between phloem samples of *P. halepensis*, *P. sylvestris*, *P. pinaster* and *P. pinea* from stands located in central Portugal would be detectable and concluded that their patterns, as our results also show, were differentiable and distinct. Additionally, GCxGC studies on needles of 11 different adult pine species located in central Portugal were performed, where a successful discrimination of species and chemical characterization were achieved (Mateus *et al.*, 2010). Moreover, Mumm *et al.* (2003; 2004) had analysed 35- to 45-year old *P. sylvestris* needle volatiles and 14-year old *P. nigra* branch volatiles by GC-MS that were produced after egg deposition or artificially wounding. They found that these damages significantly affect the quantitative composition of the samples headspace and that the volatile blend of egg-laden *P. nigra* differs from the odour of egg-carrying *P. sylvestris* both quantitatively and qualitatively.

These results allow indicate that, although it is possible to distinguish between species, new volatile production in response to PWD doesn't appear to be affected at 3 hai or it cannot be discriminated in a simple GC run. These secondary metabolism compounds, despite their action as primary defence against pathogenic agents, aren't detected by this technique as early as the timepoint in study.

4.3. *Suppression Subtractive Hybridization*

Pathogens commonly present pathogen-associated molecular patterns (PAMPs) to their hosts, which trigger activation of nonspecific basal defence mechanisms, like transcription of thousands of stress-related genes (Hu *et al*., 2009). Resistance to microbial infections requires transcription of a wide range of genes encoding regulatory and antimicrobial proteins, which are likely to impact the state of chromatin and DNA modifications (Dhawan *et al*., 2009).

SSH using isolated RNA plays an important role in molecular investigations, as it allows to compare at a molecular level two samples for which one wants to find transcriptional differences, in this case, between *P. pinaster* and *P. pinea*, 3 hai with *B. xylophilus* geographical isolate HF.

One of the most important factors in any successful SSH experiment is the quality of the RNA samples used. The protocol for RNA extraction was the one followed by Le Provost *et al*. (2007), which had previously been optimized for RNA extraction in pine xylem tissues. Other more expedite RNA protocols, such as the Qiagen RNeasy Plant Extraction protocol had been previously tested in our laboratory and were found unsuitable for this application. The extracted total RNA obtained using the optimized method was quantified by spectrophotometry and purity evaluated by A_{260}/A_{280} ratio (1,8>2,0). The results suggested that the extracted RNAs were of suitable quality to proceed with the experiments (Table 3).

Table 3 – RNA quality and quantity by spectrophotometric analysis

Sample	**Yield (ng/µl)**	**A_{260}/A_{280}**
Tester RNA	708	2,0
Driver RNA	180	2,0

The SSH protocol can use either total RNA or mRNA as starting material. Initially, mRNA was chosen as the starting material. To this end, mRNA isolation was conducted using the Oligotex mRNA Midi kit (Qiagen). However, the final quantity of poly A+ RNA obtained was too low when compared to the amounts required in PCR-Select^{TM} cDNA Subtraction Kit (Clontech) (as 2 µg was necessary and about 16 ng was obtained for both samples).

Therefore, total RNA ended up being the chosen starting material for this protocol. First and second strands of cDNA were synthesized using the SMARTer^{TM} PCR cDNA Synthesis Kit (Clontech). Then, it was necessary to optimize the cycle number for Long Distance-PCR in pine nucleic acid material. The optimal cycle number should be between 18-20 cycles. As shown in Figure 7, 21 cycles was the optimal number of PCR cycles since after 24 the product started to be inhibited.

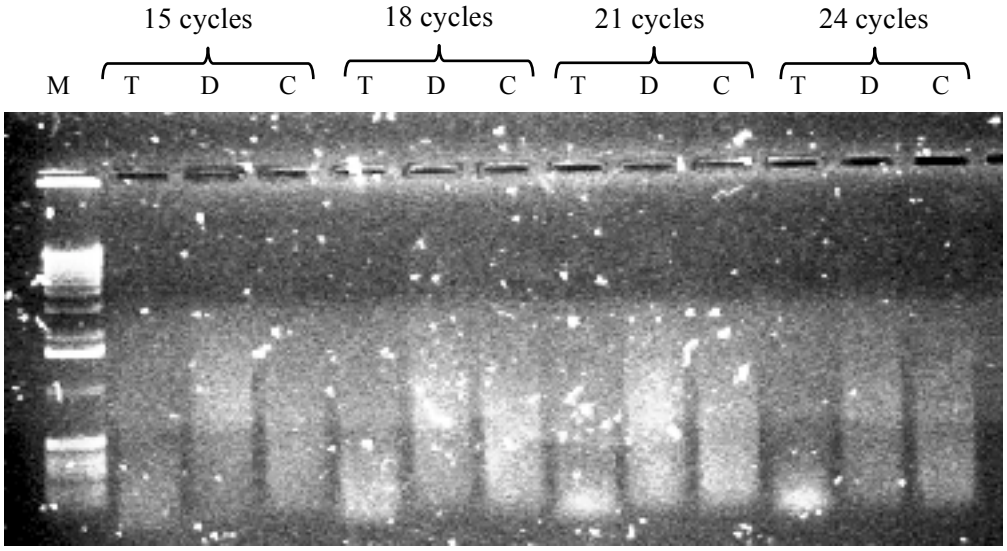

Figure 7 - Optimization of LD-PCR cycles. Lane M – Marker 1kb; Tester cDNA (T), Driver cDNA (D) and Control (C) after 15, 18, 21 and 24 LD-PCR cycles. Samples were run on a 2% agarose/gel red, 1X TAE buffer.

The PCR products were purified by column chromatography as suggested by the manufacturer's instructions; however this method wasn't very viable since almost all product appeared to be lost, as shown by the very faint smears in Figure 8.

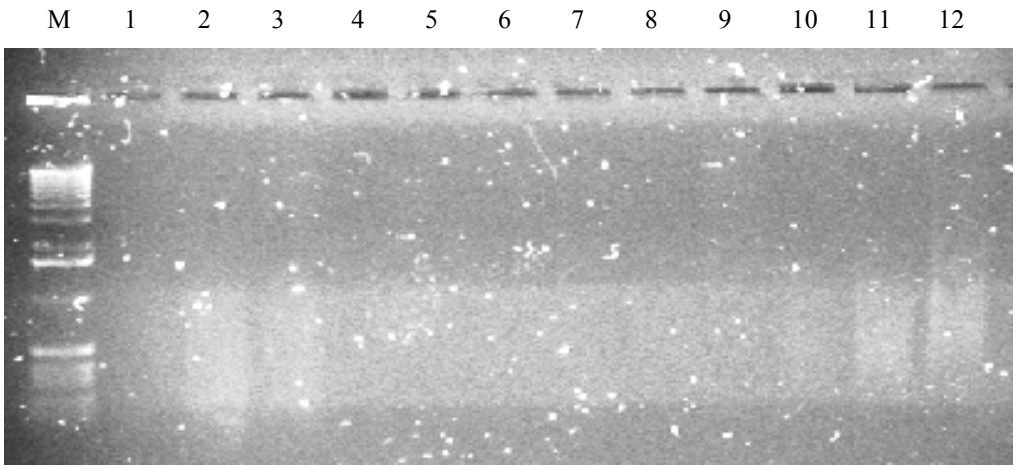

Figure 8 - Column chromatography products. Lane M - Marker 1 kb; Lanes 1, 2, 3, 4 –Tester cDNA; Lanes 5, 6, 7, 8 – Driver cDNA; Lanes 9, 10, 11, 12 – Control cDNA. Samples were run on a 2% agarose/gel red, 1X TAE buffer.

In consequence, ds cDNA was again synthesized but this time purified using illustra GFX PCR DNA Purification kit (GE Healthcare) which was more reliable as a purification method for plant material then the advised by the SSH kit from Clontech (Figure 9). Sperotto *et al*. (2007; 2009) also used this system with good results. As evidenced in Figure 9 the smear is now visible in the electrophoresis gel. After this change in the protocol, a quantity of 400 ng/µl with 1,8 ratio of A_{260}/A_{280} was obtained for each sample. An additional modification was made, as a second elution of the product was done at the final purification step, in order to retrieve any significant amount of sample trapped in the membrane.

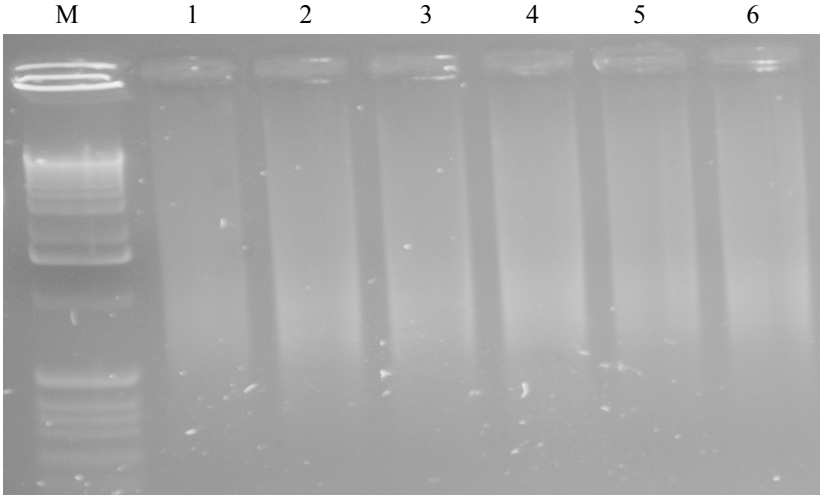

Figure 9 - Purified ds cDNA samples using the GFX PCR DNA purification method. Lane M - Marker 1 kb; Lanes 1, 2, 3 - Tester cDNA; Lanes 4, 5, 6 - Driver cDNA. Samples were run on a 2% agarose/gel red, 1X TAE buffer.

Following the PCR-Select™ cDNA Subtraction Kit instructions, adaptors 1 and 2R (please see Materials and Methods) were ligated to the purified ds cDNA. For this, a previous step of RsaI digestion was necessary, to create blunt-ended ds cDNA fragments that are required for adaptor ligation. At this point, two tester populations were created with different adaptors ligated; these adaptors have stretches of identical sequence to allow annealing of the PCR primer once the recessed ends have been filled in.

Two hybridizations were then performed, where an excess of driver was added to the tester populations and samples were heat denaturated, allowed to anneal and the concentration of high and low abundance sequences equalized. During the hybridizations, differentially expressed sequences are significantly enriched, the two primary hybridization samples are mixed together and, after this, only the remaining equalized and subtracted ss tester cDNAs can re-associate and form ds hybrids. Fresh denatured driver cDNA was added to molecules of tester with different ends (corresponding to the sequences of adaptors 1 and 2R) and different annealing sites for the nested primers on their 5' and 3' end were created in the differentially expressed tester sequences.

This population of molecules was subjected to PCR and only the desired differentially expressed sequenced were exponentially amplified. A secondary PCR amplification was performed to further reduce any background PCR products. The resulting products were analysed by electrophoresis (Figure 10).

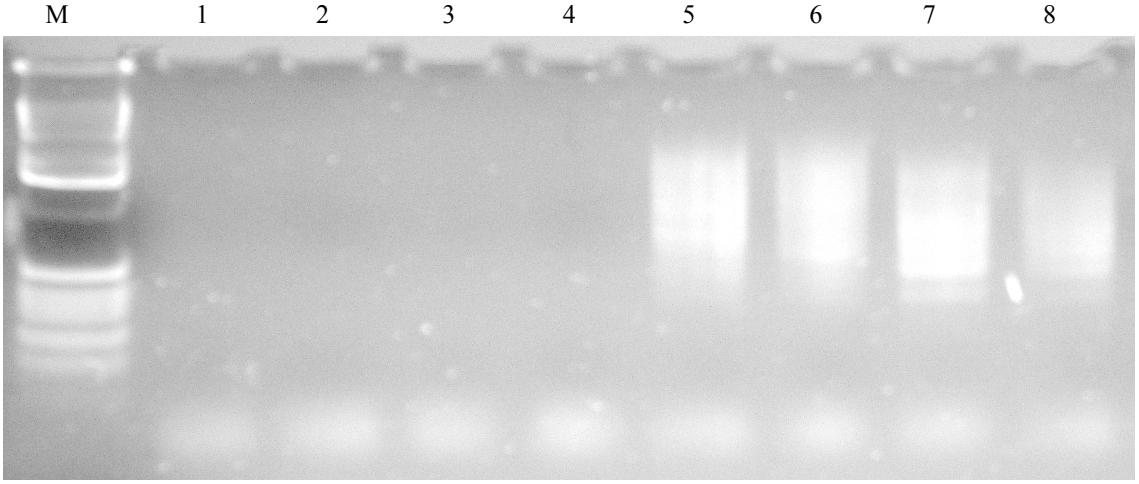

Figure 10 - Electrophoresis of secondary PCR products. Lane M – Marker 1 kb; Lanes 1, 2 – Forward subtraction; Lanes 3, 4 – Reverse subtraction; Lanes 5, 6 – Unsubtracted tester; Lanes 7, 8 – Unsubtracted driver. Samples were run on a 2% agarose/gel red, 1X TAE buffer.

As can be seen in Figure 10, the expected smears from 0.2-1 kb are only visible in the unsubtracted samples; this suggests that either there was no product amplified or the yield of the cDNA synthesis was very low. Although the results were unclear, the procedure was continued with cloning of sequences of forward and reverse subtractions and unsubtracted tester and driver with TOPO TA Cloning Kit. On the resulting selective agar plates (Figure 11) a total of 90 white colonies were selected, grown and sent for sequencing to Macrogen (Korea), and although the number of colonies was significantly higher in the unsubtracted samples, there was also growth in the subtraction corresponding plates.

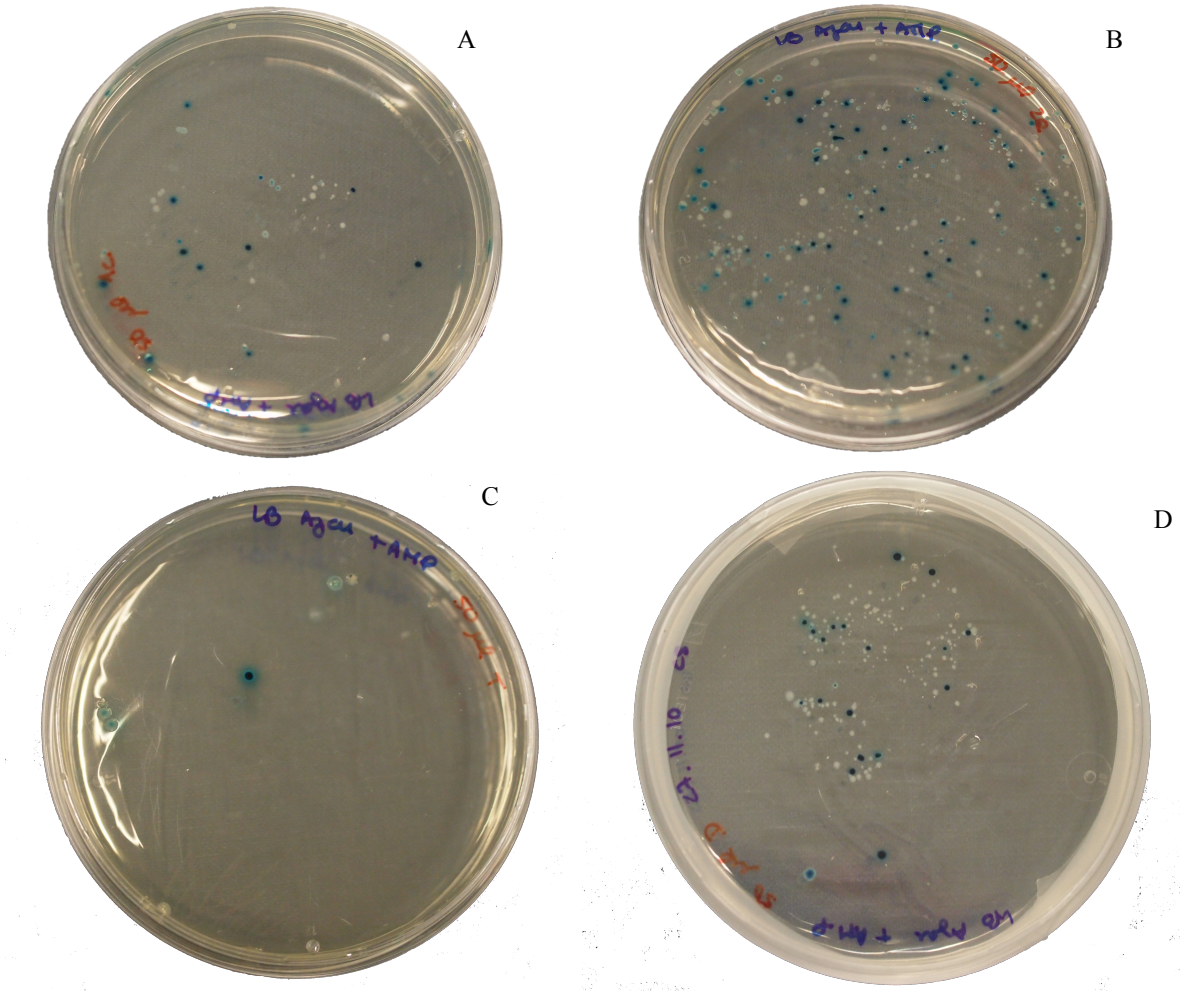

Figure 11 - Blue/white screening for target clones. A total of 50 µl of cloning product were plated on AmpxGal selective plates. A) Unsubtracted Tester; B) Unsubtracted Driver; C) Forward Subtraction; D) Reverse Subtraction.

Of the 90 colonies, 19 were not possible to sequence, possibly due to insufficient sample quantity; 12 were selected from the forward subtraction and 33 clones were selected from the reverse subtraction; also 12 from the unsubtracted tester and 15 from the unsubtracted driver were selected for cloning.

Differentially expressed genes (forward and reverse subtracted samples)

Sequences obtained by forward subtraction got no significant hits in BlastN and BlastX searches of NCBI (Table 4); from the 33 sequences obtained by reverse subtraction, only 5 had hits in NCBI search databases, all of them similar to putative histones H4 of *Picea* spp. (Table 5). Histone H4 is one of the proteins belonging to chromatin, which is present in the nucleosome, contains flexible N-terminal tails, and undergoes diverse post-translational modifications (Yan *et al*., 2007).

It has been reported that cyst nematode feeding-site construction interferes with the normal nuclear biology of the host cell, by triggering repeated cycles of DNA endoreduplication (Elling *et al*., 2007). Several histone-related enzymes have been reported to have a particularly important role in plant defence against pathogens, namely: 1) histone deacetylase 19 (HDA19), that represses transcription, as they remove the acetyl group from core histones, resulting in a closed chromatin configuration, which results in a global repressor molecule (Mach, 2008); 2) histone methyltransferase, where histone H3 lysine 4 methylation is associated with transcriptional activation and trimethylation associated with gene silencing (Berr *et al*., 2010; Pontvianne *et al*., 2010; Tittel-Elmer *et al*., 2010); and 3) histone monoubiquitination1, that may be implicated in the control of the cell cycle and seed dormancy (Dhawan *et al*., 2009).

It is, therefore, an interesting observation that the gene that was found to be differentially expressed in *Pinus pinea* when compared to *Pinus pinaster* codifies for a probable H4 histone protein. This could suggest that H4 expression is one of the molecular players involved in the lower susceptibility of *P. pinea* when compared to the more susceptible species *P. pinaster*.

Putatively expressed genes by *P. pinaster* (unsubtracted tester)

Of the 12 colonies that were grown and sent for sequencing to Macrogen, only one had no significant hit in the NCBI database. Of the other 10 classified proteins from the expressed genes in *P. pinaster* (Table 6), a putative alfa tubulin was isolated, similar to *Picea wilsonii* species. Tubulins belong to a multigene family in plants and are involved in the formation of earlywood microfibril angle (González-Martínez *et al.*, 2007) and have a role in microtubule and spindle organization (Dryková *et al*., 2003). There is still no published data that makes a direct correlation between alfa tubulin expression and nematode infection; however, one can hypothesise that, as nematodes feed of the xylem cells and disrupt wood formation, newly synthesised tubulin could be required to re-synthesise nematode-destructed feeding sites.

Also, a possible cytosolic Fe-S protein was identified, and these are usually involved in numerous metabolic processes such as isomerization and dehydration reactions, and serve as electron carriers in various redox reactions (Kispal *et al.*, 1999). As described before, dehydration is one of the symptoms of nematode infection (Tan *et al.*, 2005). In fact, two of the genes isolated from the unsubtracted tested were related to water or oxidative stress. The first one, a likely cytochrome oxidase subunit I, is reported to be abundantly expressed during drought and/or heat stress (Rizhsky *et al*., 2004). The second one, although it was unknown in NCBI database, was found to have a conserved domain (see Table 8, Ut-10) belonging to a putative thioredoxin. The TRX family plays an important role in the defence against oxidative stress hence it acts as a signalling molecule in plants through its redox-regulation properties (Jacquot *et al*., 1997). Oxidative stress is an important response of pine trees when infected by the PWN and is a result of, for example, release of metabolites by the action of enzymes in nematode saliva and the host's secondary metabolites, that exposes plant cells to highly toxic oxygen species (Shin *et al*., 2009).

A putative translation elongation factor-1, an eukaryotic elongation factor 1 alpha (EF1α), was also identified, that has the role to present amino acyl-tRNA to the A site of ribosomes. When in stress conditions (e.g. hypoxia), it appears to form non-functional complexes with polysome-associated mRNA and prevents peptidyl synthesis and translocation. So, the overexpression of these factors such as EF1α might favour expression of glycolytic enzymes, whose activity must be sustained for the surviving of plant cells with O_2 lack. (Hochachka *et al*., 1996)

A gene expressing phenylalanine ammonia lyase (PAL) was identified; it is responsible for the production of secondary metabolites in the phenylpropanoid pathway. This is activated in the incompatible interaction between pathogen-host trees as a defence gene and was proposed to be involved in synthesizing precursors for lignification, a possible nematode resistance process (Mauch-Mani and Slusarenko, 1996).

Other four genes were isolated, but couldn't be classified in NCBI databases. Therefore, their conserved domains were identified in order to obtain further information (Table 8). Genes of (or related to) RNA recognition motif (RRM), also known as RNA-binding domain or ribonucleoprotein domain were expressed (Ut-1) and they usually form complexes with RNAs and/or proteins. These are present in post-transcriptional events, like pre-mRNA processing, splicing, alternative splicing, mRNA stability, translation regulation and degradation (Maris *et al*., 2005).

One other unknown sequence was identified, and based on its conserved domain analysis, it was found that it may be a xyloglucan endotransglycosylase (XET) and was detected amongst *P. pinaster* expressed genes (Ut-6). This enzyme cleaves preferably xyloglucan polymers in plant cell walls and has the ability to re-ligate to a different acceptor chain, thereby controlling wall extensibility (Eckard, 2004; Fry *et al.*, 1993). The induction of rapid cell wall elongation is one of the primary responses of plant tissues to auxin and this is thought to arise from an increase in the mechanical extensibility of the cell wall (Catalá *et al*., 1997).

The conserved domains also allowed us to identify a non-specific lipid-transfer protein type 1 (Ut-7) that has the ability to catalyse the transfer of lipids between membranes (Torres-Schumann *et al*., 1992). These protein have also been implicated in plant defence and their ability to inhibit bacterial and fungal pathogens have been described since they are distributed in high concentrations over exposed surfaces and they seem to be frequently expressed in response to infection with pathogens (Blilou *et al*., 2000).

Table 4 - Sequence similarities of subtracted tester, *P. pinaster*, cDNAs (T) selected following SSH

Seq.	Size (bp)	Protein/DNA similarity	Origin of similar sequence	Blast X		Blast N	
				Acc No. of similar sequence	Similarity	Acc No. of similar sequence	Similarity
T1	305	-	-	-	-	-	-
T2	203	-	-	-	-	-	-
T3	127	-	-	-	-	-	-
T4	153	-	-	-	-	-	-
T5	950	-	-	-	-	-	-
T6	708	-	-	-	-	-	-
T7	178	-	-	-	-	-	-
T8	587	-	-	-	-	-	-
T9	770	-	-	-	-	-	-
T10	3	-	-	-	-	-	-
T11	70	-	-	-	-	-	-
T12	32	-	-	-	-	-	-

Table 5 - Sequence similarities of subtracted driver, *P. pinea*, cDNAs (D) selected following SSH

Seq.	Size (bp)	Protein/DNA similarity	Origin of similar sequence	Blast X		Blast N	
				Acc No. of similar sequence	Similarity	Acc No. of similar sequence	Similarity
D1	507	Histone H4	*Picea sitchensis*	ABK21562.1	103 aa	EF082188.1	3e-44 (79%, 615 bp)
D2	519	Histone H4	*Picea sitchensis*	P35057.2|H4_SOLLC	7e-38 (98%, 103 aa)	EF081475.1	9e-41 (82%, 654 bp)
D3	203	-	-	-	-	-	-
D4	950	-	-	-	-	-	-
D5	950	-	-	-	-	-	-
D6	862	-	-	-	-	-	-
D7	950	-	-	-	-	-	-
D8	950	-	-	-	-	-	-
D9	950	-	-	-	-	-	-
D10	950	-	-	-	-	-	-
D11	950	Histone H4	*Picea glauca*	NP_001077477.1	5e-19 (74%, 86 aa)	BT115137.1	6e-30 (74%, 612 bp)
D12	950	Histone H4	-	XP_001768205.1	4e-05 (61%, 103 aa)	-	-
D13	947	-	-	-	-	-	-
D14	872	GJ17461-like (H4)	*Picea glauca*	XP_002686736.1	2e-04 (80%, 138 aa)	BT105693.1	1e-18 (72%, 914 bp)
D15	617	-	-	-	-	-	-
D16	11	-	-	-	-	-	-
D17	159	-	-	-	-	-	-
D18	950	-	-	-	-	-	-
D19	950	-	-	-	-	-	-
D20	867	-	-	-	-	-	-
D21	842	-	-	-	-	-	-
D22	950	-	-	-	-	-	-
D23	950	-	-	-	-	-	-
D24	950	-	-	-	-	-	-
D25	950						
D26	819						
D27	840	-	-	-	-	-	-
D28	3	-	-	-	-	-	-
D29	197	-	-	-	-	-	-
D30	844	-	-	-	-	-	-
D31	950	-	-	-	-	-	-
D32	147	-	-	-	-	-	-
D33	479	-	-	-	-	-	-

Table 6 - Sequence similarities of unsubtracted tester, *P. pinaster*, cDNAs (Ut) selected following SSH

Seq.	Size (bp)	Protein/DNA similarity	Origin of similar sequence	Blast X		Blast N	
				Acc No. of similar sequence	Similarity	Acc No. of similar sequence	Similarity
1c-1	864	Unknown	*Picea sitchensis*	ABK24055.1|	1e-107 (89%, 299 aa)	EF084744.1	0.0 (92%, 1227 bp)
1c-2	866	EST, clone 14-5 JM E7(20)	*P. pinaster*	B8LK84.1|DRE2 1_PICSI	9e-14 (100%, 285 aa)	FN257026.1	4e-132 (100%, 481 bp)
1c-3	867	Alfa tubulin (TUA1)	*Picea wilsonii*	XP_002981542.1	1e-146 (99%, 449 aa)	EU268195.1	0.0 (95%, 1356 bp)
1c-4	950	-	-	-	-	-	-
1c-5	191	Cytochrome oxidase	*Ixodes ricinus?*	AAP93883.1	7e-81(100%, 158 aa)	AY327035.1	8e-05 (100%, 477 bp)
1c-6	867	Unknown	*Picea sitchensis*	ADE77904.1	2e-93 (86%, 274 aa)	BT124669.1	0.0 (91%, 1122 bp)
1c-7	538	Unknown	*Pinus taeda*	ABK20926.1	1e-55 (95%, 123 aa)	FJ104302.1	3e-163 (93%, 598 bp)
1c-8	866	Translation elongation factor-1	*Picea sitchensis*	CAC27139.1	1e-149 (95%, 444 aa)	BT071362.1	0.0 (93%, 1872 bp)
1c-9	609	Phenylalanine ammonia-lyase	*P. pinaster*	ACS28225.2	1e-102 (97%, 718 aa)	AY641535.1	0.0 (99%, 2708 bp)
1c-11	866	Unknown	*Picea sitchensis*	ABK21322.1	3e-62 (100%, 120 aa)	EF676404.1	1e-48 (85%, 639 bp)
1c-12	867	Unknown	*Picea glauca*	ABK25472.1	3e-71 (84%, 367 aa)	BT102048.1	0.0 (89%, 1644)

Table 7 - Sequence similarities of unsubtracted driver, *P. pinea*, cDNAs (Ud) selected following SSH

Seq.	Size (bp)	Protein/DNA similarity	Origin of similar sequence	Blast X		Blast N	
				Acc No. of similar sequence	Similarity	Acc No. of similar sequence	Similarity
2c-1	866	Unknown mRNA	*Picea sitchensis*	ABK21925.1	5e-36 (96%, 77 aa)	EF082569.1	0.0 (88%, 717 bp)
2c-2	867	Unknown	*Picea glauca*	-	-	BT116785.1	6e-09 (84%, 1746 bp)
2c-3	866	Clavata-like receptor	*Picea glauca*	ABF73316.1	1e-108 (83%, 998 aa)	DQ530597.1	0.0 (84%, 2997 bp)
2c-4	865	Unknown	*Picea glauca*	ABK23251.1	1e-136 (90%, 299 aa)	BT103799.1	0.0 (89%, 1272 bp)
2c-5	537	mRNA up-regulated during drought stress	*P. pinaster*	-	-	AJ309123.1	1.0e-100 (89%, 348 bp)
2c-6	705	Unknown	*Picea sitchensis*	ABK22226.1	5e-76 (87%, 160 aa)	BT071582.1	1.0e-168 (86%, 945 bp)
2c-7	866	N14 matrix protein	*Pinctada maxima*	BAA90539.1	140 aa	AB032612.1	3e-05 (93%, 814 bp)
2c-8	519	Unknown	*Picea glauca*	ABK24266.1	1e-45 (97%, 535 aa)	BT115938.1	0.0 (92%, 1907 bp)
2c-9	518	s-adenosyl methionine synthetase 2	*P. taeda*	ACO57105.1	3e-35 (100%, 94 aa)	AY874759.1	0.0 (98%, 541 bp)
2c-10	511	CopC	*Pseudomonas fluorescens*	ACL13563.1	1e-35 (96%, 127 aa)	EU927287.1	1.0e-109 (86%, 4741 bp)
2c-11	783	Unknown	*Picea glauca*	ACN40917.1	1e-126 (91%, 437 aa)	BT107125.1	0.0 (89%, 1540 bp)
2c-12	412	Unknown	*Picea sitchensis*	ABK21554.1	93 aa	EF082180.1	0.0 (85%, 614 bp)
2c-13	442	Unknown	*Picea sitchensis*	ABK20902.1	1e-29 (100%, 60 aa)	EF677978.1	2e-94 (88%, 445 bp)
2c-14	863	Unknown	*Picea sitchensis*	ABK22836.1	248 aa	EF083489.1	5e-47 (82%, 1215)
2c-15	689	Anonymous locus	*P. pinaster*	-	-	FN257100.1	3e-128 (87%, 365)

Finally, a likely FMN-dependent alpha-hydroxyacid was also isolated from *P. pinaster* (Ut-11), an oxidizing enzyme located in the peroxisomes that plays an important role in photorespiration where it oxidizes glycolate to glyoxylate (Stenberg and Lindqvist, 1997). Photorespiration also provides protection against abiotic stress conditions caused by drought (Reumann and Weber, 2006), which can be related to the water stress caused by PWN.

Putatively expressed genes by *P. pinea* (unsubtracted driver)

One of the classified proteins obtained in *P. pinea* (Table 7) was a putative clavata-like receptor, an important regulator of cellular processes and protein kinase that might be involved in the tailor of defence response against pathogens (Chisholm *et al*., 2006). Also, a probable N14 matrix protein was isolated, similar to *Pinctada maxima*, a mollusc shell component, which lead us into thinking that was not a host gene (Kono *et al*., 2000).

Other important PWD-related expressed gene was a possible s-adenosyl methionine synthetase 2, as it plays a role in the biosynthesis of polyamines and ethylene (Peleman *et al.*, 1989). Enhanced ethylene production during PWD is another physiological symptom known, as it results from nematode movement that ultimately results in embolism in tracheids (Fukuda, 1997). Also, as pathogenic infection of plants increases the respiration rate, ethylene causes increases, and the ethylene-induced increase is further potentiated by increased oxygen, acting as a defence mechanism (Ecker and Davis, 1987).

A likely copper resistance protein, CopC - blue copper, was also isolated, and these proteins are predicted to be involved in antioxidant defence, by reducing reactive oxygen species produced in response to pathogens and/or other stresses (Verica *et al*., 2004). A mRNA up-regulated during drought stress was also identified. Besides protein accumulation and activation, mRNA accumulation is another level of gene expression and it has been demonstrated that, when in water-deficit, mRNA content increases (Bray, 2002).

Ten other *P. pinea* expressed proteins were unclassified in NCBI databases. In order to obtain further information, their conserved domains were studied and it was possible to identify 6 of the referred proteins (Table 8). A possible gene expressing a protein belonging to the 4F5 protein family (Ud-1) was identified. The functions of these proteins are still

unknown, however it's clear that they are rich in aspartate, glutamate, lysine and arginine, and are ubiquitously expressed (Scharf *et al*., 1998).

Similarly to one protein obtained in *P. pinaster* (Ut), a probable RNA recognition motif was identified (Ud-4) that, as already mentioned, are present in post-transcriptional events. Also, a sequence expressing a putative protein belonging to Class-II DAHP synthetase family was isolated (Ud-8); these are involved in the first step of the shikimate pathway that may occur exclusively at the genetic level (Herrmann and Weaver, 1999). The resulting product of this pathway, chorismate, is the common precursor for phenylalanine/tyrosine or tryptophan (Zucko *et al*., 2010; Hu *et al*., 2009), which are important compounds in plant defence. Again, phenylalanine related proteins were also isolated in *P. pinaster*. Shikimate is also involved in the phenylpropanoid pathway, crucial in lignin production (Wagner *et al*., 2007).

A gene sequence encoding a protein similar to one belonging to DUF231 *Arabidopsis* proteins was isolated (Ud-11). Although its function is unknown, several members of this family are believed to be co-expressed with other cell wall-related genes, specifically in cellulose biosynthesis (Bischoff *et al*., 2010). Other putative ribonucleoprotein related protein, Sm-like protein (Ud-12), was identified in *P. pinea*, which are involved in a variety of RNA processing events (Séraphin, 1995). Finally, a probable NifU-like protein (Ud-14), a scaffold protein, crucial regulator of many key signalling pathways was also detected (Yabe *et al*., 2004).

Table 8 – Proteins and respective conserved domain found in Unsubtracted tester (Ut), Unsubtracted driver (Ud) and Subtracted driver (D)

Seq.	Protein	Conserved Domain
Ut-1	Unknown	RNA recognition motif, involved in post-transcriptional gene expression processes including mRNA and rRNA processing, RNA export and RNA stability
Ud-4	Unknown	
Ut-2	EST, clone 14-5 JM E7 (20)	Cytokine-induced anti-apoptosis inhibitor 1, Fe-S biogenesis; functions in cytosolic Fe-S protein
Ut-3	Alpha tubulin (TUA1)	Major component of microtubules; can be divided into three regions, the amino-terminal nucleotide-binding region
Ut-5	Cytochrome oxidase subunit I	Heme-copper oxidase subunit I; catalyse the reduction of O2 and simultaneously pump protons across the membrane

Table 8 – Proteins and respective conserved domain found in Unsubtracted tester (1c), Unsubtracted driver (2c) and Subtracted driver (D) *continued*

Seq.	Protein	Conserved Domain
Ut-6	Unknown	Xyloglucan endotransglycosylase; cleave and relegate xyloglucan polymers in plant cell walls via transglycosylation mechanism
Ut-7	Unknown	Non-specific lipid-transfer protein type 1; facilitate the transfer of fatty acids, phospholipids, glycolipids and steroids between membranes; also play a key role in the defense of plants against pathogens
Ut-8	Translation elongation factor-1 alpha	Eukaryotic elongation factor 1 alpha; interacts with the actin of the eukaryotic cytoskeleton and may thereby play a role in cellular transformation and apoptosis
Ut-9	Phenylalanine ammonia-lyase	Member of the Lyase class I-like superfamily of enzymes; catalyzes the conversion of L-phenylalanine to E-cinnamic acid
Ut-10	Unknown	TRX family; plays an important role in the defense against oxidative stress by directly reducing hydrogen peroxide and certain radicals and by serving as a reductant for peroxiredoxins
Ut-11	Unknown	FMN-dependent alpha-hydroxyacid oxidizing enzymes; key enzyme in photorespiration where it oxidizes glycolate to glyoxylate
Ud-1	Unknown	4F5 protein family; short proteins that are rich in aspartate, glutamate, lysine and arginine; found to be ubiquitously expressed
Ud-3	Clavata-like receptor	Protein kinases, catalytic domain; PK regulate many cellular processes including proliferation, division, differentiation, motility, survival, metabolism, cell-cycle progression, cytoskeletal rearrangement and immunity
Ud-8	Unknown	Class-II DAHP synthetase family; aldolase enzymes that catalyse the first step of the shikimate pathway
Ud-9	s-adenosyl methionine synthetase 2	S-adenosylmethionine synthetase, C-terminal domain; responsible for polyamines and ethylene production
Ud-10	CopC	Copper resistance protein CopC; bacterial blue copper protein that binds 1 atom of copper per protein molecule. Along with CopA, CopC mediates copper resistance by sequestration of copper in the periplasm.
Ud-11	Unknown	Duf231 Arabidopsis proteins of unknown function
Ud-12	Unknown	Sm-like protein; associate with RNA to form the core domain of the ribonucleoprotein particles involved in a variety of RNA processing events;
Ud-14	Unknown	NifU-like domain; alignment of the carboxy-terminal domain; biochemical function unknown
D1	Histone H4	Histone H4; bind to DNA and wrap the genetic material into “beads on a string” in which DNA is wrapped around small blobs of histones at regular intevals;
D2	Histone H4	
D11	Histone H4	
D12	Histone H4	
D14	Histone H4	

As presented in Figure 12, 58% of the sequences were clustered into "no significant homology" and other 21% into "unclassified protein", as their hit in BlastN was 'unkown'. However, as can be seen in Figure 13, 40% of the unclassified proteins were found to be defence related by the analysis of their conserved domains.

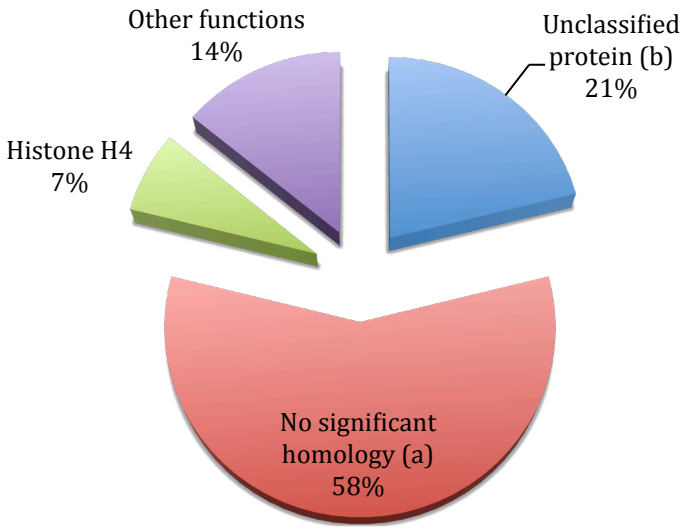

Figure 12 – Functional categorization of induced genes. All 71 genes were functionally annotated using BlastN and BlastX at NCBI, and categorizated based on GO annotation. Percentages of genes in each category are also presented. (a) These genes had no significant hits in BlastN and BlastX searches of NCBI. (b) These genes matched proteins annotated as 'unknown protein'.

Therefore, these results showed that defence-related genes are expressed in the early stage of the response. Particularly genes involved in PWD, like ethylene production and oxidative stress related genes are triggered in the first three hours after infection, in *P. pinaster* and *P. pinea*.

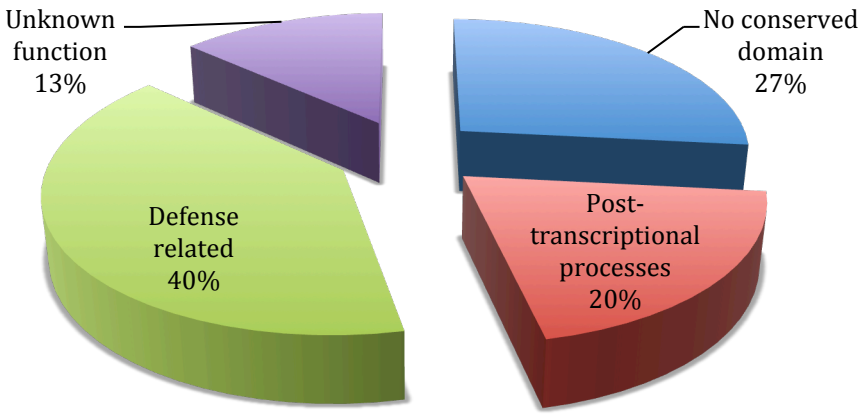

Figure 13 – Conserved domain information about the 15 genes annotated as "unknown protein" in NCBI databases.

As referred in the introduction chapter, little is known about *Pinus* spp. genome. The most significant hits (30%) showed that the sequences expressed had more homology with *Picea* spp. (Figure 14), like *P. abies*, *P. glauca* and *P. sitchensis*, all of them reported to be susceptible to the disease or an alternative PWN host (Zhao *et al.*, 2008).

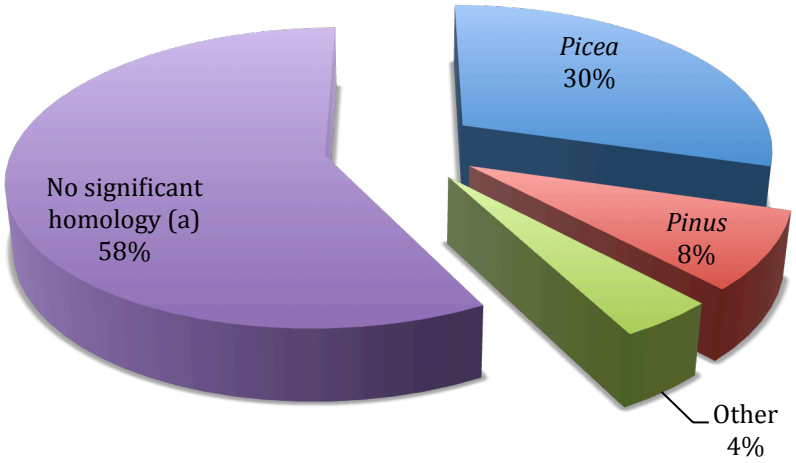

Figure 14 – Genus characterization of induced genes. (a) These genes had no significant hits in BlastN and BlastX searches of NCBI.

Although these results reveal important facts about PWD and their consequences in the plant at a molecular level, namely the time when the response is triggered, some limitations were critical during the procedure. The most important one was its requirement of large quantities of mRNA per samples which was difficult to obtain with high purity, since

cleaning protocols represent a loss of almost 50% of material (Dr.ª Conceição Egas. personal communication).

Another limitation was that differentially expressed cDNAs obtained from SSH were cDNA fragments digested by the RSA restrictive enzyme, and needed to be amplified to get the full length sequences (Liang *et al*., 2004). Therefore, this is a long and time consuming protocol that, at each step in data production, introduces variability leading to potential errors. To avoid negative results due to individual differences, samples were pooled from four seedlings per treatment.

Although SSH has the advantage of conducting normalization and subtraction in a single step, it fails too overcome the intrinsic disadvantages of generating truncated cDNA fragments (Dai *et al*., 2009). Moreover, this method requires an efficient method for obtaining large quantities of high quality RNA (Xu *et al*. 2006). However, this is hard when using recalcitrant tissues (like conifer tissues) that contain high concentrations of polyphenols, polysaccharides and other secondary metabolites (Wang *et al*., 2010). The most common extraction methods are optimized for animal tissues, especially for those rich in RNase, and are unsuitable for plants. The most classical technique to extract RNA from pine samples is based on CTAB extraction buffer (Provost *et al*., 2007); however this method also presented some difficulties, as it didn't allow to effectively remove the referred compounds. Moreover, it requires a LiCl precipitation that consists in an overnight incubation, which contributed to the long time needed to perform this work.

As Verica *et al*. (2004) showed in their work, several genes encoding a variety of transcription factors, transcriptional regulators and biosynthesis of secondary metabolites related to defence response were isolated. These responses can participate in the induction of wound response, antimicrobial and antifungal defence and antioxidant defence.

Therefore, the resistance or susceptibily to infection of a particular plant genotype within a species is determined by interwined layers of defence, including both constitutive barriers and inducible reactions (Thordal-Christensen, 2003). Also, the control of inducible reactions emphasizes the importance of understanding the mechanisms by which plants both perceive environmental signal and transmit them through the cellular machinery to activate adaptive responses (Afzal *et al*., 2008).

5. CONCLUSIONS

In the current study, where the early response of physiological changes were analysed, a tendency for water content decrease from control to nematode inoculated plants was demonstrated. The same was true about chlorophyll concentration, which had already been shown by Fukuda (1997) to have a close relationship with water content.

Regardless of these symptoms being usually associated to the later stages of the disease, a clear reduction in water content and chlorophyll concentration was detected 3 hours after infection. This is a new, revealing result that shows that nematode infection provokes a quick and immediate damage to the infected trees. Also, *P. pinea* and *P. pinaster* showed different physiological responses to the disease. As Futai (2003) demonstrated, host susceptibility to PWD varies between tree species, which might be reflected in their phylogenetic relationships in the genus.

With gas chromatography we concluded that at 3 hours after infection it wasn't possible to make a clear distinction between control and nematode-inoculated trees. On the other hand, as already demonstrated by Santos *et al*. (2006), a clear distinction between species was successfully achieved; although this method was only performed in order to see the volatile pattern distribution for each treatment, it allowed us to see that as early as 3 hai, *de novo* volatile compounds produced as a defence mechanism against the nematode, weren't detectable.

The results of this work show that as early as 3 hours after infection a molecular response is triggered in nematode inoculated pine trees. Several genes were isolated and the majority of them were defence-related. The differential expression studies had as a result the expression of putative histones that are involved in several processes, for example, repressing of transcription, gene silencing and control of cell cycle and seed dormancy.

Genes associated with oxidative stress were also detected in *P. pinaster* and in *P. pinea* samples, that are very important in PWD since as previously referred water stress accelerates symptom development; genes expressing enzymes related to cell wall synthesis were found, like hypothetical PAL and putative class-II DAHP synthetase, which are involved in the synthesis of lignin, a cell wall component believed to be responsible - when in high

quantity – to a PWD-resistance mechanism; also related to this process, probable elongation factor 1α and XET were isolated.

Still correlated with the disease, a possible s-adenosyl methionine synthetase 2 was identified, which is involved in ethylene production, a key component of the disease process, as its production is enhanced with nematode movement; also, specific defence-related genes were expressed, like lipid-transfer proteins. Post-transcriptional events-related genes were also identified.

The fact that 58% of the isolated sequences didn't have a significant homology in the NCBI database reveals that publicly available databases have very little information on the *Pinus* spp. genome sequences. Further studies are needed to unveil information at a molecular level that can be important for a better understanding of the mechanisms of PWD. Significant homology was found with *Picea* spp., that has been studied for its susceptibility to the nematode, and that has much more publicly available genome sequence information.

It is important to consider that this study was conducted in very young trees, that have a faster development of the disease symptoms when compared to older, mature ones (Zhao *et al*., 2008). However, all of these works showed that the early phase of infection is very important. We detected not only an activation of defence response at a molecular level, but also a quick physiological and metabolical stress response to the disease in the studied trees. These insights also showed that there is a distinctive reaction between the studied species, *P. pinaster* and *P. pinea*, to the disease, which is in concordance with the different susceptibility reported to these species.

6. FUTURE WORK

For a better understanding of this disease, it's imperious that genomic knowledge is gathered about the host and the nematode itself. Transcript profiling plays an important role in annotating and determining gene functions and next-generation sequencing technologies allow one to generate large scale ESTs efficiently (Daurelio *et al*., 2010; Guo *et al*., 2010). A study of *P. pinaster* and *P. pinea* transcriptome using 454 pyrosequencing is already on course in the laboratory, and will give a positive and elucidative insight about PWD and its mechanisms. It will also allow us to identify the cloned sequences that were put thus far in the "unclassified" and "unidentified" categories after the homology searches in NCBI databases.

It would be important to construct a new SSH subtracted library in order to isolate and enrich our current library with more specifically up-regulated genes due to the infection by the PWN. Moreover, real-time qRT-PCR could be added to verify the differences in the relative abundance of transcripts of genes from the inoculated and non-inoculated trees. Real-time PCR permits the monitoring of the reaction during the amplification process, eliminating the need for additional steps and thus reducing the risk of contamination (Huang, L. *et al*., 2010). Plus, only nematode-inoculated plants were used for library construction due to the high cost implication of the SSH technique, therefore it would be interesting if the new subtraction could include water inoculated plants as control for this process.

In what concerns the physiological changes during PWD, more symptoms could be studied, namely lignin content. As stated before, a gene expressing a very interesting enzyme was isolated, PAL, which is key in phenylpropanoid metabolism and leads to the synthesis of this cell-wall component. It has been reported that the difference in response to the disease between certain species and the ability to resist to the infection maybe due to lignin content, therefore making this a component of interest.

In this study, the analysis of phenolic compounds produced during the disease was only based on GC. A confirmatory analysis that would allow identification of the individual volatiles could be done, using GC-MS technique, coupled with an increased number of samples.

Furthermore, a time course trial could be performed, testing the variation in response during the different stages of PWD. This could contribute to the identification of disease indicators and of the best time to block symptom development.

Summing up, pyrosequencing could be done to better understand pine and nematode transcriptome; real-time qRT-PCR could be added to the SSH protocol in order to verify the differences in the relative abundance of transcripts of genes; more physiological changes during the disease could be taken into consideration, namely lignin content; phenolic compound production should be further analysed by GC-MS technique and a time-course trial could be structured to study symptom development during the different stages of disease.

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